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WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Twenty-seventh Report

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WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Geneva, 2–8 December 1975

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WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Twenty-seventh Report

The WHO Expert Committee on Biological Standardization met in Geneva from 2 to 8 December 1975. The meeting was opened on behalf of the Director-General by Dr D. Tejada-de-Rivero, Assistant Director-General, who spoke of the emphasis that was now being placed on the application of standards and of the increased use of the biological standardization programme in a more practical way, especially in the developing countries. He asked the Committee to pay particular attention to the type of meetings needed in the future and to consider whether the format used at the twenty-sixth meeting of the Committee, in which a single group of substances was discussed, should be repeated.

GENERAL

The Committee was of the opinion that in future all requests from various organizations for the establishment of international biological standards, reference preparations, and reference reagents should be channelled through the World Health Organization. The Committee considered that priority should be given to substances used in prophylaxis, therapy or diagnosis of human diseases and for which, by use of international standards and reference preparations, a designation of activity could be made in quantitative terms. There may be occasions, however, when the Committee will have to consider biological reference reagents for use in qualitative procedures, and for which the assignment of a unit of measurement is not applicable. In such cases a careful selection will have to be made of those substances that could serve as international reference reagents on the basis of their need and value in diagnosis. On the other hand, the Committee considered that the establishment of standards, reference preparations or reference reagents for biological substances used only for research purposes should not be the concern of the Committee.

There are also occasions when WHO technical units ask the Committee to give international recognition to certain biological materials which those units have themselves obtained and investigated. Previous examples of such occasions are the diagnostic sera for viruses and lepto-

spires. The Committee considered that this was a valuable service and one that should be continued in future. The biological substances concerned must be of the high quality required by the Committee.

The Committee was of the opinion that its work should continue to advance on a broad front and where a special subject called for treatment in depth this could be done by holding a workshop prior to a meeting of the Expert Committee. As a result of such a workshop, the formulation of requirements and the establishment of international standards, reference preparations, or reference reagents could be recommended to the Expert Committee. The Committee agreed that such a procedure might be adopted in the case of blood products and related substances before its next meeting. The Committee concluded, therefore, that the procedure followed at its twenty-sixth meeting of devoting the whole agenda to a single special subject should not be repeated.

SUBSTANCES

ANTIBIOTICS

1. Doxycycline

The Committee noted the results [1] of the collaborative study referred to in its twenty-fourth report.[2] The Committee also noted that in accordance with the authorization in its twenty-fifth report[3] the National Institute for Biological Standards and Control, London, on the basis of the results of the collaborative assay had established the International Reference Preparation of Doxycycline and with the agreement of the participants in the collaborative assay had defined the International Unit for Doxycycline as the activity contained in 0.0011494 mg of the International Reference Preparation of Doxycycline.

2. Neomycin

The Committee noted the results [4] of the collaborative study of the proposed second International Reference Preparation of Neomycin. The Committee also noted that in accordance with the authorization in its

[1] Unpublished working document WHO/BS/75.1099.
[2] WHO Technical Report Series, No. 486, 1972, p. 9.
[3] WHO Technical Report Series, No. 530, 1973, p. 5.
[4] Unpublished working document WHO/BS/75.1097.

twenty-fifth report,[1] the National Institute for Biological Standards and Control, London, had established the second International Reference Preparation of Neomycin, and with the agreement of the participants in the collaborative assay had defined the International Unit for Neomycin as the activity contained in 0.0012903 mg of the second International Reference Preparation of Neomycin.

3. Candicidin

The Committee noted the results [2] of biological, chemical and physical studies of the preparation of candicidin referred to in its twenty-third report.[3] The chemical analyses showed that this preparation of candicidin was representative of candicidin currently being produced. Stability studies showed that it had retained its potency for five years. Its suitability for use in the assay of levorin [4] had not been determined because samples of current production of levorin were not available. The Committee decided that the preparation of candicidin was suitable to serve as an international biological preparation of candicidin.

The Committee requested the National Institute for Biological Standards and Control, London, to arrange a collaborative assay of the candicidin preparation, and to include if possible a sample of levorin representative of material currently being used therapeutically. The Committee also authorized the National Institute for Biological Standards and Control to establish the material as the international reference preparation of candicidin on the basis of the results of the collaborative assay and to define the international unit with the agreement of the participants.

4. Trichomycin [5]

The Committee noted that the preparation of trichomycin referred to in its twenty-third report [3] had been included in the study of the proposed international reference preparation of candicidin.[2] The composition of the trichomycin differed qualitatively and quantitatively from candicidin.

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[1] WHO Technical Report Series, No. 530, 1973, p. 6.

[2] Unpublished working document WHO/BS/75.1108.

[3] WHO Technical Report Series, No. 463, 1971, p. 12.

[4] WHO Technical Report Series, No. 384, 1968, p. 12.

[5] Trichomycin is now known by the international non-proprietary name of hachimycin.

The Committee was informed that trichomycin was no longer in widespread use and agreed that there was at present no need for an international reference preparation.

5. Minocycline

The Committee noted the results [1] of the collaborative assay referred to in its twenty-fifth report and established the preparation assayed as the International Reference Preparation of Minocycline and defined the International Unit for Minocycline as the activity contained in 0.0011587 mg of the International Reference Preparation of Minocycline.

6. Virginiamycin

The Committee was informed that as yet unpublished specifications for virginiamycin had been approved by a national pharmacopoeial commission. The analytical methods specified require two chemical reference preparations for determining the two main components, virginiamycin M and virginiamycin S, and also a biological reference preparation of virginiamycin for use in assays of microbiological activity.

The Committee was informed also that virginiamycin was used only to a limited extent in human and veterinary medicine but was widely used as an animal feed additive for growth promotion. A number of countries had approved the extensive veterinary use of this antibiotic on the condition that it would not be used in man.

The Committee agreed, therefore, that since the main use of virginiamycin was as a feed additive for livestock there was no need for the WHO Secretariat to arrange for the provision of international reference materials or specifications for this antibiotic.

7. Bleomycin

The Committee noted that in accordance with the request in its twenty-third report [2] the WHO Secretariat had collected information [3] on bleomycin and other antitumour antibiotics that demonstrated a need for international reference materials to be used in the control of these substances. The information also indicated that although there was currently no assay for antitumour activity in use by national control

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[1] Unpublished working document WHO/BS/75.1104.

[2] WHO Technical Report Series, No. 463, 1971, p. 13.

[3] Unpublished working document WHO/BS/75.1101.

laboratories, assays for antibacterial activity were part of national specifications for certain antitumour antibiotics.

The Committee was informed that international specifications for bleomycin and doxorubicin, another antitumour antibiotic, were being prepared and that whether biological reference preparations or chemical reference substances, or both, will be needed would depend on the nature of the specifications. In the case of bleomycin, however, it was likely that the specifications would include a test requiring the use of a biological reference preparation. The Committee therefore requested the National Institute for Biological Standards and Control, London, to obtain a quantity of bleomycin suitable for this purpose.

8. Tetracycline and Oxytetracycline

The Committee noted that international specifications [1] for tetracycline hydrochloride and oxytetracycline hydrochloride based on chemical and physical requirements were being prepared. The Committee also noted that the establishment of WHO chemical reference substances [1] needed in procedures for controlling the proportions of degradation products in tetracycline had been authorized [2] by the WHO Expert Committee on Specifications for Pharmaceutical Preparations and that work to accomplish this was in progress at the WHO Collaborating Centre for Chemical Reference Substances at Solna, Sweden.

9. Spectinomycin

The Committee noted that in accordance with the request in its twenty-fourth report [3] the National Institute for Biological Standards and Control, London, had ascertained that there was a need for an international reference preparation of spectinomycin [4] and had obtained a quantity of spectinomycin suitable for this purpose.

The Committee was informed that the material had been distributed into ampoules. The Committee therefore established this material as the International Reference Preparation of Spectinomycin and requested the National Institute for Biological Standards and Control to arrange a limited collaborative assay since the material was part of a batch that had been used to provide the only two existing national standards.

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[1] Unpublished working document WHO/BS/75.1118.

[2] WHO Technical Report Series, No. 567, 1975.

[3] WHO Technical Report Series, No. 486, 1972, p. 11.

[4] Unpublished working document WHO/BS/75.1103.

The Committee authorized the National Institute for Biological Standards and Control to define the international unit with the agreement of the participants in the collaborative assay.

10. Tobramycin

The Committee noted that there was a need for an international reference preparation of tobramycin.[1] The Committee therefore requested the National Institute for Biological Standards and Control, London, to obtain suitable material and to arrange a collaborative assay.

11. Antibiotic Sensitivity (Susceptibility) Discs

The Committee was informed of the history of WHO activities concerned with antibiotic microbic sensitivity testing. The WHO Expert Committee on Antibiotics[2] had recommended that for general clinical use the method for testing microbes isolated from patients for their susceptibility or resistance to various antibiotics should be one in which filter paper discs impregnated with antibiotics are applied to the surface of inoculated culture media in plates.

The Expert Committee on Antibiotics had concluded that careful control of the uniformity of antibiotic content in the discs was of the utmost importance to the success of such tests. In 1972 the WHO Secretariat conducted a survey of the use and national quality control of antibiotic susceptibility discs, the results of which showed that the disc method of antibiotic susceptibility testing was used in all the 23 countries replying to the survey. There was also an overwhelming indication that requirements for the quality of discs were needed, and since there were only a few countries for which national requirements existed the Committee requested the WHO Secretariat to investigate the possibility of arranging for the formulation of international requirements.

BLOOD PRODUCTS

12. Human Thrombin

The Committee noted the report[3] of the collaborative assay of the proposed international standard for human thrombin. Ten laboratories

[1] Unpublished working document WHO/BS/75.1102.

[2] WHO Technical Report Series, No. 210, 1961, p. 12.

[3] Unpublished working document WHO/BS/75.1096.

had taken part in the study and all the assays of relative potency included were statistically valid. The Committee noted that the slope of the dose/response line of the bovine thrombin did not differ significantly from that of the human thrombin and that both human and bovine thrombin preparations could be assayed in terms of the proposed international standard for human thrombin.

The Committee noted that stability tests on the proposed international standard showed that after a period of 10 years at −20°C the potency loss would be negligible. The Committee, therefore, established this material as the International Standard for Human Thrombin and defined the International Unit of Human Thrombin as the activity contained in 0.0853 mg of the International Standard for Human Thrombin.

ANTIGENS

13. Carcinoembryonic Antigen

The Committee noted a report [1] on the need for an international standard or reference preparation of carcinoembryonic antigen (CEA) and that a collaborative assay had taken place of a freeze-dried preparation that gave straight and parallel dose/response lines when assayed against several other preparations of CEA in a number of laboratories. The preparation 73/601 had been established as the British Standard for Carcinoembryonic Antigen and had been offered to the World Health Organization for consideration of adoption as an international standard.

The Committee was informed of the existence of other similar preparations that were in use in other countries. The Committee asked the WHO Secretariat, in conjunction with the National Institute for Biological Standards and Control, London, to ensure that no conflict in the assignment of the unitage would arise should the British Standard for CEA be established as an international reference preparation. In the event of there being no such conflict the Committee requested the National Institute for Biological Standards and Control to establish this preparation as the international reference preparation of carcinoembryonic antigen and to assign 100 international units of CEA to each

[1] Unpublished working document WHO/BS/75.1110.

ampoule. One international unit of CEA would be defined as 0.0236 mg of the international reference preparation of CEA.

14. Alphafetoprotein

The Committee noted the results [1] of the collaborative assay of alphafetoprotein in a preparation of human cord serum 72/225. This preparation was supplied by the International Agency for Research on Cancer, Lyons, and had been freeze-dried at the National Institute for Biological Standards and Control, London. It had been shown to be particularly suitable to serve as an international standard for alphafetoprotein.

The Committee established the preparation as the International Standard for Alphafetoprotein and defined the International Unit of Alphafetoprotein as 0.0013991 mg of the International Standard for Alphafetoprotein.

15. *Clostridium welchii* (*C. perfringens*) Beta and Epsilon Toxoids

The Committee noted the results [2] of an international collaborative assay of the proposed international standards for beta and epsilon toxoids prepared from a type C and a type D strain of *Clostridium welchii* (*C. perfringens*) respectively. The Committee also noted that a significant dose/response relationship had been demonstrated in the majority of assays of the beta component but that the relationship was less significant for the epsilon component. Estimates of potency of test preparations using the proposed standards varied widely within and between laboratories. The Committee suggested that further work with these preparations should include the use of the flocculation test.

The Committee agreed that there was not sufficient evidence to recommend the establishment of these preparations as international standards but considered that their establishment as international reference preparations would be useful in the control of test systems. Accordingly, the Committee established the two preparations as the International Reference Preparation of *Clostridium welchii* (*C. perfringens*) Beta Toxoid and the International Reference Preparation of *Clostridium welchii* (*C. perfringens*) Epsilon Toxoid, without assigning any unitage to them.

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[1] Unpublished working document WHO/BS/75.1121.

[2] Unpublished working document WHO/BS/75.1122.

16. Diphtheria Toxoid, Plain

The Committee noted the report [1] from the Statens Seruminstitut, Copenhagen, on the proposed replacement of the International Standard for Diphtheria Toxoid, Plain. This document had been circulated to the Expert Advisory Panel on Biological Standardization and other experts and as no objections had been received the Director-General had established the Second International Standard for Diphtheria Toxoid, Plain, and had defined the International Unit of Diphtheria Toxoid, Plain as the activity contained in 0.10515 mg of the Second International Standard for Diphtheria Toxoid, Plain.

17. Diphtheria Toxoid, Adsorbed

The Committee noted that stocks of the International Standard for Diphtheria Toxoid, Adsorbed, were almost exhausted and that there was a need for their replacement.[2] The Committee reviewed the preliminary data concerning two preparations of diphtheria toxoid, adsorbed, that had been obtained by the Statens Seruminstitut, Copenhagen, and agreed that those preparations should be included in an international collaborative assay arranged by the Statens Seruminstitut. The Committee authorized the Statens Seruminstitut on the basis of the results of the collaborative assay to establish the more suitable preparation as the second international standard for diphtheria toxoid, adsorbed, and with the agreement of the participants to assign an international unit to it.

18. Purified Protein Derivative of Bovine Tuberculin

The Committee noted the proposal [3] for an international standard for purified protein derivative (PPD) of bovine tuberculin. The proposal had been made because of the specific antigenic difference that had been shown to exist between human and bovine PPD preparations. The Committee agreed that there was a need for an international standard for bovine PPD in addition to the international standard for mammalian PPD, which was in fact human PPD. The Committee requested the Central Veterinary Laboratory, Weybridge, England, to obtain suitable material and to arrange a collaborative assay with a view to establishing a standard for PPD of bovine tuberculin. The Committee suggested that in carrying out this study it was necessary to include tests in infected cattle.

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[1] Unpublished working document WHO/BS/74.1075.
[2] Unpublished working document WHO/BS/75.1105.
[3] Unpublished working document WHO/BS/75.1125.

19. *Clostridium botulinum* Type B Toxin

The Committee noted the report[1] indicating that *Clostridium botulinum* type B toxin was heterogeneous. The Committee requested the Statens Seruminstitut, Copenhagen, together with the Chiba Serum Institute, Japan, to investigate this matter in relation to the International Standard for *Clostridium botulinum* Type B Antitoxin.

MISCELLANEOUS SUBSTANCES

20. International Reference Preparation of Opacity

The Committee noted a document[2] that had been circulated to members of the Expert Advisory Panel on Biological Standardization concerning a proposed replacement of the third International Opacity Reference Preparation. The Committee was informed that no adverse comments had been received and that the Director-General had established the preparation proposed in the document as the fourth International Opacity Reference Preparation.

The Committee noted the report[3] of the Symposium on the Measurement of the Number of Organisms in Bacterial Vaccines, held jointly by WHO and the International Association of Biological Standardization, at which it had been agreed that suspensions of glass particles in water were unsuitable as reference preparations because of instability. The Symposium had recommended that a plastic, which also simulated the optical properties of bacterial suspensions and which was stable, should be adopted as the international opacity reference preparation.

The Committee, after being informed of the results of a collaborative assay in which a particular sample of plastic prepared in the form of a cylindrical rod was compared with the International Opacity Reference Preparation, agreed that this would be a more suitable substance for an international opacity reference preparation. The Committee adopted the plastic as the fifth International Reference Preparation of Opacity and assigned 10 International Units of Opacity to the plastic, which for convenience was prepared in the form of a rod in order that the opacity of bacterial suspensions held in tubes of the same internal diameter as the external diameter of the rods might be compared with it.

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[1] Unpublished working document WHO/BS/75.1107.

[2] Unpublished working document WHO/BS/75.1074.

[3] Unpublished working document WHO/BS/75.1119.

The Committee discontinued the fourth International Opacity Reference Preparation.

ANTIBODIES

21. Anti-D Immunoglobulin

The Committee noted the results [1] of a collaborative study arranged by the National Institute for Biological Standards and Control, London, as requested in the twenty-fourth [2] report. The collaborative study was made to assess the suitability of a preparation of anti-D immunoglobulin to serve as an international reference preparation. The Committee also noted that, by the use of this preparation as a reference, automated haemagglutination, manual haemagglutination and the isotopic procedure all gave similar estimates of the relative potency of other test preparations. However, the automated haemagglutination technique gave greater precision. The Committee agreed that an international standard for anti-D immunoglobulin would serve a useful purpose and noted that the National Institute for Biological Standards and Control was making further studies to determine whether the preparation could be calibrated in terms of the International Unit already assigned to the International Standard for Anti-Rh_0 (anti-D) incomplete blood-typing serum established in 1966.

22. Anti-*Echinococcus* Human Serum

The Committee noted the results [3] of the studies on the proposed international reference preparation of anti-*Echinococcus* serum carried out as requested in its twenty-third report [4] by the Statens Seruminstitut, Copenhagen. The Committee agreed that the establishment of a reference preparation for diagnostic purposes would be useful in the control of antigens used in diagnostic tests. The Committee therefore established the anti-*Echinococcus* human serum as the International Reference Reagent of Anti-*Echinococcus* Human Serum.

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[1] Unpublished working document WHO/BS/75.1111.
[2] WHO Technical Report Series, No. 486, 1972, p. 16.
[3] Unpublished working document WHO/BS/75.1106.
[4] WHO Technical Report Series, No. 463, 1971, p. 23.

23. Anti-*Brucella abortus* Serum

The Committee noted the results [1] of a collaborative assay and data obtained from national control authorities showing that the agglutination test and the complement-fixation test measure two different biological activities in anti-*Brucella abortus* sera. For this reason, and on the basis of the collaborative assay, the Committee assigned to the second International Standard for Anti-*Brucella abortus* Serum, which had originally been established on the basis of agglutination tests, a unitage of 1000 International Units of agglutinating activity and 1000 International Units of complement-fixing activity per ampoule.

REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

24. Requirements for Yellow Fever Vaccine

The Committee discussed the proposed revised Requirements for Yellow Fever Vaccine.[2] The Committee agreed that, until it was possible for the majority of manufacturers to obtain a source of eggs free from avian leucosis viruses (ALV), requirements demanding freedom from these extraneous agents could not be written since they might reduce the availability of vaccine and create an unacceptable public health risk. Nevertheless the amendments proposed to the Committee were sufficiently significant to warrant detailed consideration. Changes of particular importance involved the assay of vaccine potency and the procedure for evaluating viral neurotropism. The Committee adopted these amendments but rather than have two documents (the original requirements and the amendments), the Committee agreed that the revised requirements should be published in their entirety (see Annex 1).

The Committee agreed that it was desirable to encourage the production of yellow fever vaccine free from ALV, mycoplasma and other agents. It would be helpful to obtain a seed devoid of these contaminants that could be distributed by WHO to manufacturers. The Committee asked the Secretariat to investigate the possibility of obtaining such a seed. The Committee also requested the Secretariat to obtain a reference preparation that could be used in the tests for viscerotropism and neurotropism as well as for the determination of potency of the vaccine.

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[1] Unpublished working document WHO/BS/75.1124.

[2] Unpublished working document WHO/BS/75.1100 Rev. 1.

25. Requirements for Meningococcal Polysaccharide Vaccine

The Committee discussed the Requirements for Meningococcal Polysaccharide Vaccine,[1] which had been presented by a WHO Study Group on Cerebrospinal Meningitis Control,[2] and noted that this was one of a new class of vaccines consisting of purified components of bacteria and the first of the class for which international requirements had been written. The Committee noted that, because of the nature of these new products, proper control involves the use of chemical as well as biological assays. The Committee recognized that the rapid pace of meningococcal vaccine research can be expected to lead to improvements in the polysaccharide products and possibly to the development of vaccines containing immunizing antigens derived from other meningococcal components. Nevertheless, the formulation of international requirements is desirable since the vaccines currently being produced are effective and are being widely used. In terms of technical evolution, one area of likely progress involves the potential for increasing the molecular weight of the polysaccharides. A correlation exists between molecular weight and immunogenicity for man; consequently when vaccines containing polysaccharides of higher molecular weight (i.e., lower values of the chromatographic distribution constant, K_D) become generally available it will be desirable to reduce the upper limits of the K_D value specified in the International Requirements.

The Committee adopted the Requirements for Meningococcal Polysaccharide Vaccine (see Annex 2) and agreed that because of the new technology involved in the production and control of the vaccine it would be useful to include an appendix describing methods found acceptable for polysaccharide purification, the estimation of molecular size, the assay of *O*-acetyl groups, and the determination of the bactericidal antibody content of human sera.

26. Specifications of Tests Used in the Requirements for *Brucella abortus* Strain 19 Vaccine (Live—for Veterinary Use)

The Committee noted the detailed description[3] prepared by the National Animal Disease Laboratory, Ames, IA, USA, and the Central Veterinary Laboratory, Weybridge, England, of the tests specified in the

[1] Unpublished working document WHO/BS/75.1112.

[2] WHO Technical Report Series, No. 588, 1976.

[3] Unpublished working document WHO/BS/75.1114.

Requirements for *Brucella abortus* Strain 19 Vaccine (Live—for Veterinary Use). The Committee was informed that details of these tests would be made available by the WHO Secretariat on request and they are annexed to this report (see Annex 3).

27. Requirements for *Brucella melitensis* Strain Rev. 1 Vaccine (Live—for Veterinary Use)

The Committee noted that in accordance with the request in its twenty-third report,[1] requirements for *Brucella melitensis* strain Rev. 1 vaccine (live—for veterinary use) had been drafted.[2]

The Committee requested the WHO Secretariat to circulate the draft for comment to the members of the Expert Advisory Panel on Biological Standardization as well as to producers of the vaccine and to other experts in the subject.

28. Requirements for Rickettsial Products

The Committee noted that rickettsial vaccines, particularly those for louse-borne typhus and Q fever, were used in some parts of the world.[3] However, the Committee was informed that the control of typhus involved many procedures, only one of which was the use of vaccine in target populations in endemic areas. The Committee agreed that the formulation of requirements for rickettsial vaccines was not justified at present but that this question should be kept under review.

29. Requirements for Rubella Vaccine

The Committee noted a suggestion that there was a need for international requirements for rubella vaccine.[4] The Committee agreed that since rubella vaccine was now used in many countries and was made by several manufacturers, international requirements for rubella vaccine would serve a useful purpose. The Committee asked the WHO Secretariat to take the necessary steps to draw up such requirements.

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[1] WHO Technical Report Series, No. 463, 1971, p. 24.
[2] Unpublished working document WHO/BS/75.1115.
[3] Unpublished working document WHO/BS/75.1116.
[4] Unpublished working document WHO/BS/75.1117.

MISCELLANEOUS

30. Manuals

The Committee discussed the need for manuals on the manufacture, standardization and control of vaccine in addition to the " Guide to the provision of technical facilities " published in its twenty-second report.[1] The Committee agreed that for those vaccines used extensively in the WHO expanded programme on immunization much could be achieved by having guidelines for the production and testing of vaccines.

Furthermore the Committee agreed that there was a need to promote the establishment of basic vaccine control laboratories in the developing countries in order that the health authorities in those countries might have a better understanding of the products being used in their health programmes.

The Committee agreed that the WHO Secretariat should take steps to provide special manuals and guidance on the establishment of vaccine control laboratories.

31. Human Blood Products and Related Substances

The Committee noted a list of human blood products and related substances[2] proposed for its consideration. The Committee agreed that the technology for the standardization of these substances had developed to a great extent and that uniformity in that field was desirable. The Committee requested the WHO Secretariat to obtain information on the need for international standards for human blood products and related substances.

32. List of Biological Substances

The Committee was informed of the amendments that would be needed to bring the List of Biological Substances (published in 1975) up to date. The Committee considered that the substances available from each international laboratory should be in alphabetical order within classified subjects, and although this requirement would necessitate a complete redrafting of the publication it was nevertheless important that the redrafting should be done. Ideally the list should be reviewed

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[1] WHO Technical Report Series, No. 444, 1970, p. 71, Annex 3.

[2] Unpublished working document WHO/BS/75.1123.

each year. The Committee agreed that if that could not be done each report of the Committee should have an annex listing the changes made since its previous meeting. The Committee suggested the possibility of storing the list on a computer programme in order that rapid revisions could be made.

ACKNOWLEDGEMENTS

The Committee wished to record its thanks to the following members of the WHO Secretariat for their special contributions to its deliberations: Dr M. Abdussalam, Chief, Veterinary Public Health; Dr P. Brès, Chief, Virus Diseases; Dr W. C. Cockburn, Director, Division of Communicable Diseases; Dr B. Cvjetanovic, Chief, Bacterial Diseases; Dr J. Guld, Tuberculosis; Dr M. L. Tarizzo, Virus Diseases; and Dr W. Wieniawski, Pharmaceuticals.

Annex 1

REQUIREMENTS FOR YELLOW FEVER VACCINE [a]

(Requirements for Biological Substances No. 3)
(Revised 1975)

INTRODUCTION

Requirements for Yellow Fever Vaccine (Requirements for Biological Substances No. 3) were formulated by a WHO Study Group in 1958.[1] They were based on the " Standards for the Manufacture and Control

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[a] Prepared by the following members of the WHO Secretariat :
Dr D. W. Barry, Director, General Virology Branch, Division of Virology, Bureau of Biologics, Food and Drug Administration, Bethesda, MD, USA (*Consultant*) ; Dr P. Brès, Chief, Virus Diseases, WHO, Geneva, Switzerland ; Dr B. L. Elisberg, Director, Division of Pathology, Bureau of Biologics, Food and Drug Administration, Bethesda, MD, USA (*Consultant*) ; Professor C. Hannoun, Unité d'Ecologie Virale, Institut Pasteur, Paris, France (*Consultant*) ; Dr D. Magrath, Division of Viral Products, National Institute for Biological Standards and Control, London, England (*Consultant*) ; Dr F. T. Perkins, Chief, Biological Standardization, WHO, Geneva, Switzerland ; Dr Y. Robin, Director, Institut Pasteur, Dakar, Senegal (*Consultant*).

[1] WHO Technical Report Series, No. 179, 1959, Annex 1.

of Yellow Fever Vaccine " adopted by the Standing Committee on Health of UNRRA in 1945.[1] The requirements embodied the proposed revisions of the UNRRA standards recommended by the first WHO Expert Committee on Yellow Fever Vaccine,[2] and they applied to vaccine prepared from a suitable strain of yellow fever virus which was intended to be given by subcutaneous injection. Conformity with the requirements has since been the basis for WHO approval of yellow fever vaccine used for vaccination and revaccination against yellow fever in connexion with certification for the purposes of international travel,[3] and such approval has been given only to vaccine made using suitable seed derived from the 17D strain of yellow fever virus. The requirements have been used also by national authorities for the control of yellow fever vaccine used in national immunization programmes. The twenty-second WHO Expert Committee on Biological Standardization in 1969 [4] agreed that developments in virology in general and in the manufacture and control of yellow fever vaccines in particular warranted a revision of the current requirements for yellow fever vaccine, with due consideration of both the national and international application of the requirements. The third WHO Expert Committee on Yellow Fever in 1971 [5] made specific recommendations concerning these requirements.

In 1973 an informal group of consultants made specific recommendations for studies leading to the more precise determination of virus content of the vaccine and in 1975 a further meeting of consultants considered the results of the studies and formulated the present revised set of requirements. In addition, opinions and data have been received from the following experts:

Dr B. N. Bhattacharjee, Assistant Director General, Directorate General of Health Services, New Delhi, India

Dr H. H. Cohen, Director, National Institute of Public Health, Bilthoven, Netherlands

Dr V. F. Davey, Technical Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia

Mr I. Davidson, Central Veterinary Laboratory, Weybridge, England

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[1] *Epidemiological information bulletin*, 1945, **1**, p. 365.

[2] WHO Technical Report Series, No. 136, 1957, Annex 1.

[3] *International Health Regulations (1969)*, 2nd ed., Geneva, World Health Organization, 1974 (Article 67, Section 4).

[4] WHO Technical Report Series, No. 444, 1970, p. 21.

[5] WHO Technical Report Series, No. 479, 1971, p. 51.

Professor S. G. Dzagurov, Director, Tarasevič State Institute for the Standardization and Control of Medical Biological Preparations, Moscow, USSR

Professor D. G. Evans, Director, National Institute for Biological Standards and Control, London, England

Professor P. Ionescu Stoian, Institute of State Control of Medicament and Pharmaceutical Research, Bucharest, Romania

Professor G. Heymann, Paul Ehrlich Institute, Frankfurt am Main, Federal Republic of Germany

Dr R. C. Kent, WHO Technical Officer, AFRO/ICP/LAB/001, Lagos, Nigeria

Professor A. Lafontaine, Director, Institute of Hygiene and Epidemiology, Brussels, Belgium

Dr Chr. Lucasse, Chief, Yellow Fever Research and Production Unit, Department of Tropical Hygiene, Royal Institute for the Tropics, Amsterdam, Netherlands

Professor B. Lunenfeld, Director, Institute of Endocrinology, Tel-Hashomer, Israel

Dr Harry M. Meyer, Jr., Director, Bureau of Biologics, Food and Drug Administration, Bethesda, MD, USA

Dr H. Mirchamsy, Associate Director, Razi State Serum and Vaccine Institute, Karaj, Iran

Dr R. Netter, Acting Director General, National Health Laboratory, Paris, France

Dr Margaret Pittman, Bureau of Biologics, Food and Drug Administration, Rockville, MD, USA

Dr J. Prydie, Head, Virology Quality Control, The Wellcome Research Laboratories, Beckenham, England

Dr H. Voss, Director, Yellow Fever Laboratory, Robert Koch Institute, Federal Health Office, Berlin

Professor A. E. Wilhelmi, Department of Biochemistry, Emory University, Atlanta, GA, USA

Since the formulation of the requirements in 1959 there have been many advances in technology.

Much experience has been gained in the last ten years on tests for adventitious agents and many of these would be applicable to yellow fever vaccine. In several countries the seed virus has been freed from contaminating avian leucosis viruses (ALV). Current production of vaccine in eggs derived from flocks known to be free from ALV is yielding vaccine free from detectable living contaminants. Such a vaccine produced in one country has been used in man without untoward effects for a period of seven years. Adequate supplies of embryonated eggs free from ALV, however, are not readily available to all laboratories at the moment and for this reason it has not been possible to formulate requirements for only ALV-free vaccine. The use of yellow fever vaccine for more than 30 years prepared from the 17D strain now known to contain avian leucosis viruses, has not been shown to be associated with untoward long-term reactions. Although it would be ideal to have vaccine prepared free from the contaminant viruses it is difficult to justify when so many constraints militate against such a decision. In the case of manufacture of ALV-free vaccine, however, ALV-free seed and embryos free from ALV must be used, and the vaccine must satisfy these requirements and be shown to be free from ALV by a suitable test.

In the requirements formulated in 1959 an identity test is specified which requires the provision of a reference non-immune serum. The reason for this is to ensure that the neutralization of the virus in the product under test is effected by specific antibody rather than by non-specific inhibitors that may be present in the normal serum of the human or animal species used for the production of the immune serum. In the case of the other live attenuated virus vaccines (poliomyelitis, measles, mumps, and rubella), identity is determined by the neutralization of the virus by a hyperimmune serum known to be monospecific, and a non-immune serum is not required. With yellow fewer vaccine, however, one of the difficulties in the identity test is the need to use undiluted serum since the antibody content of immune sera may not be very high ; for this reason the non-immune serum should be included as a control.

Several production laboratories have been using non-immune products shown to be free from inhibitors ; some have even been using a medium containing no serum.

Ideally the non-immune preparation should be obtained from the same animal as that used for the production of the immune serum but in any case the non-immune serum should be obtained from the same species of animal. It should also be used at the same dilution as that of the immune serum and be shown not to cause a reduction in virus titre. The provision of a single international reference non-immune serum therefore appears to be impractical and it has been withdrawn.

Many of the modern techniques in virology have been taken into consideration in the revision of the requirements for this vaccine. It has now been shown that cell culture techniques are useful in tests for identity and potency. The potency test carried out with mice can be replaced by a titration technique using cell cultures. In addition to avoiding the difficulties of maintaining a mouse colony, cell cultures form a reliable substrate for the detection of virus particles and offer the advantages of greater sensitivity and reproducibility. Some laboratories, however, may be able to obtain mice but not a constant supply of cell cultures. For this reason the titration of virus content in mouse brains has been retained, but as an alternative the titration in cell cultures may be adopted. The plaque assay technique is applicable also to the identity test, the test for viscerotropism, and the tests for measurement of the antibody response of monkeys.

A test requiring more detailed study is the monkey safety test, which should always be carried out after new virus seed pools have been prepared in order to ensure that the neurotropic properties of the new material are within safe limits. It is particularly important to carry out this test if the preparation of the seed pools includes procedures to remove ALV. The suggestion has been made that the assessment of neurotropism may be made by a histopathological technique in much the same way as for poliomyelitis vaccine. The preliminary data look promising but much work remains to be done before this technique can replace the current methods of measuring neurotropism. In the meantime, clinical observations on the effect of yellow fever viruses in monkeys can be more clearly described and are embodied in the present requirements.

In the previous requirements the test for viscerotropism related to two complementary aspects. The first aspect was that at least 90% of inoculated monkeys shall develop neutralizing antibodies and that 90% of the monkeys shall develop a viremia as shown by a mouse test. The second aspect, also covered by the mouse test, was that the viremia shall be within specified limits (" not more than 10% of the monkeys must have circulating virus exceeding 100 mouse LD_{50} in 0.03 ml serum,

and no monkey should show more than 500 mouse LD_{50} in 0.03 ml serum "). Since the development of antibodies may occur without detectable viremia, it seems unnecessary, if neutralizing antibodies are demonstrated, to require that viremia is demonstrated by the inoculation of mice. Accordingly the requirement to demonstrate viremia in 90% of the monkeys has been deleted. The titration of virus in blood, however, has been retained in order to verify that the viremia did not exceed the specified limits.

The other amendments concern the testing of the cell substrate to ensure freedom from avian pathogens able to infect man and to ensure also the stability of the vaccine, particularly when it may be used in countries with high ambient temperatures.

WHO has taken steps to obtain a suitable vaccine to serve as an international reference preparation. Such a preparation is needed to determine the precision of the potency test, whether done in mice or cell culture, and it would be useful also as a reference preparation for the neurovirulence test.

The present recommendations for requirements for yellow fever vaccine concern only those to be used for subcutaneous injection. To date there are no vaccines available for use by cutaneous scarification that will satisfy the requirements of both safety and potency set forth in this document. The recommendations are based on methods and tests currently used to ensure a safe and potent vaccine ; future revisions of these requirements will be necessary if a vaccine for cutaneous scarification is developed.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements so that, if a health administration so desires, these parts as they appear may be included in definitive national requirements. The parts of each section that are printed in small type concern points on which comments seemed desirable.

Should individual countries wish to adopt these requirements as the basis for their national regulations concerning yellow fever vaccine, it is recommended that a clause be included permitting modifications of manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority and the World Health Organization, that such modified requirements ensure a degree of safety and potency of the vaccine at least equal to those provided by the requirements formulated below.

The term " national control authority " as used in these requirements always refers to the country in which the vaccine is manufactured.

PART A :
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be *Vaccinum febris flavae*. The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 Descriptive definition

Vaccinum febris flavae shall consist of a freeze-dried preparation of viable, attenuated yellow fever virus (*Flavivirus hominis*). The preparation shall satisfy all the requirements formulated below.

1.3 International standards or reference preparations and international units

The International Reference Preparation of Anti-Yellow-Fever Serum was established in 1962. It is dispensed in ampoules containing 71.5 mg of dried immune monkey serum and has been assigned a potency of 143 International Units per ampoule; it is in the custody of the International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen. It is intended for the calibration of national reference preparations for use in the virus neutralization test. The preparation may be used also in a plaque reduction test performed in cell culture.

The establishment of an International Reference Preparation of Yellow Fever Vaccine is necessary for the evaluation of the sensitivity of titration methods and as a basis for comparison in the monkey neurovirulence test.

1.4 Terminology

Primary seed lot. A quantity of virus suspension that has been processed together and has a uniform composition. It is used for the preparation of secondary seed lots.

Secondary seed lot. A quantity of virus suspension that has been processed together, is uniform with respect to composition, and is only one passage from a primary seed lot. Material is drawn from secondary

seed lots for inoculating embryonated eggs for the preparation of vaccine.

Single harvest. A quantity of virus suspension harvested from tissues that were inoculated, incubated, and processed together.

Bulk suspension. The material prepared from one or more single harvests and before filling into final containers.

Filling lot (*final lot*). A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling and drying. A filling lot must, therefore, have been filled in one working session and have been dried together.

Reconstituted vaccine. Rehydrated vaccine ready for administration.

Mouse lethal dose 50% (*mouse* LD_{50}). The quantity of virus suspension estimated to produce fatal specific encephalitis in 50% of mice of a highly susceptible strain, four to six weeks of age, after intracerebral inoculation.

Plaque forming unit (*PFU*). The smallest quantity of virus suspension that will produce a single primary plaque in a specified monolayer cell culture.

Diluent for yellow fever virus titration. A 0.75% solution of bovine albumin (fraction V), in phosphate-buffered isotonic salt solution at pH 7.4, prepared from water purified by ion-exchange resins or by glass distillation ; or any other diluent which has been demonstrated to the satisfaction of the national control authority to be equivalent for maintaining the infectivity of the virus.

Reference immune serum. A freeze-dried preparation of monospecific serum containing yellow fever virus neutralizing antibodies. An international reference immune serum is the International Reference Preparation of Anti-Yellow-Fever Serum (see section 1.3).

2. GENERAL MANUFACTURING REQUIREMENTS

The general requirements for manufacturing establishments contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)[1] shall apply to establishments manufacturing yellow fever vaccine, with the addition of the following :

Production areas shall be decontaminated before they are used for the manufacture of yellow fever vaccine. No other infectious agents shall be introduced in the area during the period of production.

[1] WHO Technical Report Series, No. 323, 1966, p. 11, Annex 1.

The production of yellow fever vaccine shall be conducted by a separate staff, which shall consist of healthy persons who shall be examined medically at regular intervals and who, during the period of production, shall not work on other infectious agents. Steps shall be taken to ensure that all such persons in the production areas and monkey quarters are immune to yellow fever and do not excrete any micro-organisms of significance to the safety of the vaccine. Personnel working in monkey quarters shall be examined also for tuberculosis as outlined in Part A, section 2 of the Requirements for Biological Substances No. 11 (Requirements for Dried BCG Vaccine).[1]

Visitors and persons not directly concerned with the production process shall not be permitted to enter the production areas.

3. PRODUCTION CONTROL

3.1 Control of source materials

3.1.1 *Virus strains*

The virus strains used in the production of vaccine must be derived from the 17D strain of yellow fever virus, shall be identified by historical records, and shall have been shown to the satisfaction of the national control authorities to be safe and immunogenic.

> Samples of tested primary seed lots of a 17D strain of virus may be obtained for use in vaccine production by application to the World Health Organization.

3.1.2 *Tissues for virus production*

Virus for the preparation of primary and secondary seed lots and of all vaccine lots shall be grown in the tissues of chick embryos obtained from a healthy flock. Monitoring of the flock or embryos shall include at least tests for exclusion of infection by *Salmonella*, *Mycobacterium avium* and fowl pox virus. The flock must not have been vaccinated with live Newcastle disease virus vaccine.

> In some countries the national control authority requires that the tissue used for production of vaccine should be shown by suitable tests to be free from ALV, mycoplasma or other agents.

3.1.3 *Seed lot system*

The production of vaccine shall be based on the primary and secondary seed lot system. All seed lots shall be stored under conditions

[1] WHO Technical Report Series, No. 329, 1966, p. 25, Annex 1.

optimum for the stability of the strain of virus, at a temperature of −70°C or below. Primary and secondary seed lots shall not contain any human protein or added serum or antibiotics.

The seed lots shall be shown to be free from bacteria and fungi by tests similar to those applied to the final vaccine and described in section 5.2.

> In some countries the national control authority requires that the primary and secondary seed lots should be shown by suitable tests to be free from ALV, mycoplasma or other agents.

The inoculum for infecting the tissues used in the production of a vaccine lot shall be a secondary seed lot without intervening passage in order to ensure that no vaccine shall be manufactured that is more than one passage removed from a seed lot that has passed all the safety tests.

3.1.4 *Monkey safety test*

Primary and secondary seed lots from their final containers shall pass a test in monkeys for viscerotropism, immunogenicity, and neurotropism.

The monkeys shall be *Macaca mulatta* (*Macacus rhesus*) species or be of an equally sensitive and responsive species and shall have been demonstrated to be non-immune to yellow fever immediately prior to injecting the seed virus. They shall be healthy and shall not have been previously subjected to intracerebral or intraspinal inoculation. Furthermore they shall not have been inoculated by other routes with neurotropic viruses or with antigens related to yellow fever. Not less than ten monkeys shall be used for each test.

The test dose shall consist of 0.25 ml containing the equivalent of not less than 5000 mouse LD_{50} and not more than 50 000 mouse LD_{50}, as shown by a titration conducted by the method described in section 5.5.[1] The test dose shall be injected into one frontal lobe of each monkey under anaesthetic, and the monkeys shall be observed for a minimum period of 30 days.

3.1.4.1 *Viscerotropism.* The criterion of viscerotropism (as indicated by the amount of circulating virus) which must be fulfilled shall be as follows.

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[1] Each laboratory should establish the number of plaque forming units equivalent to 1 mouse LD_{50}.

Sera obtained from each of the test monkeys on the second, fourth and sixth days after inoculation shall be inoculated at dilutions of 1 : 10, 1 : 100 and 1 : 1000 either into groups of at least six cell culture vessels as specified in section 5.5 or intracerebrally in 0.03 ml aliquots into groups of at least six mice similar to those used in the potency test described in section 5.5. In no case shall 0.03 ml of serum contain more than the equivalent of 500 mouse LD_{50} and in not more than one case shall 0.03 ml of serum contain more than the equivalent of 100 mouse LD_{50}.[1]

3.1.4.2 *Test for immunogenicity.* The criterion of sufficient virus-neutralizing antibody in the sera shall be as follows.

At least 90% of the test monkeys shall be shown to become immune within 30 days subsequent to the injection of the test dose, as determined by examining their sera in one of the tests for the neutralization of yellow fever virus described below.

> In some countries it has been shown that a low dilution of some sera contains non-specific inhibitors that influence this test. There may be a requirement therefore by the national control authority for the serum to be treated to remove such inhibitory substances.

Test in mice. The French neurotopic yellow fever virus (FNV) strain shall be used. If stored in the freeze-dried state, the contents of two or more containers shall be rehydrated and pooled in an amount equal to ten times the original volume of the virus suspension in those containers. After rehydration, or after thawing if stored in the frozen state, the virus suspension shall be allowed to stand at room temperature for 15 minutes. The virus dilution to be used in the test shall then be prepared in the same diluent. This test-dilution shall be such that after addition of an equal volume of non-immune serum and after incubation at 37°C for one hour, the serum-virus mixture will contain approximately 100 mouse LD_{50} in 0.03 ml. Samples of each of the monkey sera to be tested shall be mixed with an equal volume of the test-dilution of virus, and no serum-virus mixture shall be allowed to remain at room temperature for more than 15 minutes before incubation. The serum-virus mixtures shall be incubated in a water bath at 37°C for one hour and then chilled in an ice-water bath.

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[1] Each laboratory should establish the number of plaque forming units equivalent to 1 mouse LD_{50}.

Healthy mice 4–6 weeks old and of a strain highly susceptible to intracerebral inoculation of yellow fever virus shall be distributed at random into groups of not less than six for the test and into groups of not less than 12 for the controls.

From each serum-virus mixture, 0.03 ml volumes shall then be injected intracerebrally under anaesthesia into each of at least six mice. The inoculations shall begin immediately after incubation of the serum-virus mixtures and shall be completed as expeditiously as possible. The mice shall be observed for 10 days. Only deaths occurring after the third day and considered to be specific shall be taken into account. A serum shall have passed the yellow fever virus neutralization test if more than two-thirds of the mice survive and if the LD_{50} determination shows that the test dose of virus contains more than 50 LD_{50}. A serum shall have failed the yellow fever virus neutralization test if less than one-third of the mice survive and if the LD_{50} determination shows that the test dose contains less than 200 LD_{50}. All sera that neither pass nor fail the test shall be retested until they either pass or fail by these criteria.

The following control shall be carried out on the viral suspension used in the above test.

An LD_{50} determination shall be done by preparing progressive tenfold dilutions of virus and by adding to each of a series of at least five of them an equal volume of non-immune serum. The mixtures shall be incubated and injected into mice as above except that groups of at least 12 mice shall be used for each serum-virus mixture, and the range from zero to 100% mortality of the mice shall be covered.

> If the identity (potency) of the virus strain used in the above test has not been determined it should be ascertained by the following test. Progressive fourfold dilutions should be prepared of reference immune serum in undiluted non-immune serum and to each of a series of at least six of these dilutions should be added an equal volume of the test-dilution of virus. The serum-virus mixtures should be incubated and injected into mice as described above except that groups of at least 12 mice should be used for each serum-virus mixture, and the range from zero to 100% mortality should be covered. The result of this potency determination should be compared with the known neutralizing potency of the reference immune serum previously established.

Test in cell cultures. Dilutions of at least 1 : 10 (it should be borne in mind that a dilution of 1 : 10 may contain non-specific viral inhibitors), 1 : 40 and 1 : 160 of serum from each monkey shall be mixed with an equal volume of 17D vaccine virus at a dilution that has been shown to

yield an optimum number of plaques when assayed according to the method outlined in section 5.5. The serum-virus mixtures shall be incubated in a water bath at 37°C for one hour and then chilled in an ice-water bath before adding 0.2 ml aliquots of each serum-virus mixture to each of four tissue culture plates. The plates will then be handled as described in section 5.5. In addition, ten plates will be similarly inoculated with the same amount of virus plus an equal volume of a 1 : 10 dilution of monkey serum known to contain no neutralizing antibodies to yellow fever. At the end of the observation period, the mean number of plaques in the plates receiving virus plus non-immune serum shall be compared with the mean number of plaques in the plates receiving virus plus dilutions of each monkey serum. Not more than 10% of the test monkeys shall have serum that fails to reduce the number of plaques by 50% at the 1 : 10 dilution.

3.1.4.3 *Neurotropism.* The criterion of neurotropism (as indicated by the incidence of clinical manifestations of encephalitis and by the mortality rate) shall be as follows.

The monkeys shall be examined daily by personnel familiar with clinical signs of encephalitis in primates (if necessary, the monkeys may be removed from their cages and examined for signs of motor weakness or spasticity as described in the literature [1]). Not more than 10% of the test monkeys shall develop severe signs of encephalitis such as paralysis, inability to stand when stimulated, or death. In addition, the virus under test shall not induce other signs of encephalitis (such as incomplete paralysis, incoordination tremors, lethargy, inability to stand spontaneously, motor weakness or spasticity) at a frequency greater than that induced by a laboratory reference vaccine with acceptable properties in man and monkeys. The laboratory reference vaccine shall be approved by the national control authority.

> When the WHO reference vaccine preparation becomes available it will replace the laboratory reference vaccine approved by the national control authority.
>
> Neither the onset and duration of the febrile reaction nor the nature of the symptoms and pathological findings should be such as to indicate a change in the properties of the virus.[2]

3.1.5 *Sterility tests on primary and secondary seed lots*

Each final lot of seed virus in the final containers shall pass the tests described in Part A, section 5.2 of the revised Requirements for Bio-

[1] Draper, C. C. *Journal of hygiene*, 65 : 505 (1967).

[2] Fox, J. P. & Penna, H. A. *American journal of hygiene*, 38 : 152 (1943).

logical Substances No. 6 (General Requirements for the Sterility of Biological Substances).[1]

3.1.6 *Guineapig safety test on primary and secondary seed lots*

Each final lot of seed virus in the final containers shall pass the tests mentioned in section 5.3 of the present requirements.

3.2 Production precautions

The general precautions, as formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)[2] shall apply to the manufacture of yellow fever vaccine with the addition of the following.

3.2.1 *Tests on control tissues*

If the monitoring of the flocks is not under the direct responsibility of the manufacturer, the following controls on the tissues shall be included.

Ten uninoculated embryonated eggs from the batch used for vaccine production shall be incubated under the same conditions as the inoculated eggs. At the time of virus harvest, the uninoculated eggs shall be processed in the same manner as the infected embryos and the extracted juice from the control embryos shall be shown to be free from salmonella, from *Mycobacterium avium*, and from fowl pox virus by tests approved by the national control authority.

3.2.2 *Addition of stabilizers and preservatives*

No human protein or penicillin shall be added to the virus suspension at any stage during production. If stabilizing agents are added they shall be shown to the satisfaction of the national control authorities to have no antigenic or sensitizing properties for man.

3.3 Harvesting and treatment of the bulk product before desiccation

After inoculation and incubation at a controlled temperature, only living and typical chick embryos shall be harvested. The age of the embryo at the time of virus harvest shall be reckoned from the initial

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[1] WHO Technical Report Series, No. 530, 1973, p. 49.

[2] WHO Technical Report Series, No. 323, 1966, p. 15.

introduction of the egg into the incubator and shall be not more than 12 days.

After homogenization and centrifugation, the extract of the embryonic pulp shall be kept at −70°C or colder until further processing.

3.3.1 *Test for sterility*

The extract of the embryonic pulp that forms a single harvest shall be tested for the presence of bacteria and fungi as described in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).[1]

> In some countries the national control authority requires that the vaccine should be shown by suitable tests to be free from ALV, mycoplasma or other agents.

The extract of the embryonic pulp shall be tested also for the presence of *Mycobacterium avium* by cultural methods approved by the national control authority.

4. FILLING AND CONTAINERS

The requirements concerning filling and containers in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)[2] shall apply to yellow fever vaccine.

The containers of the final vaccine shall be of neutral glass of high quality. Single- and multiple-dose containers may be used.

As soon as possible after harvesting, vaccine shall be filled into containers and freeze-dried. Containers shall be sealed under vacuum or filled with dry nitrogen.

> Failure to achieve adequate drying will result in a product that is liable to rapid deterioration even at 0°C. National control authorities may require an assay for residual moisture by an approved method.
>
> Since the yellow fever virus is extremely labile, unless there is a good seal there may be variations in titre during storage due to leakage. It is therefore important that the manufacturer should check that the sealing is satisfactory.

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[1] WHO Technical Report Series, No. 530, 1973, p. 49.

[2] WHO Technical Report Series, No. 323, 1966, Annex 1, p. 16.

The manufacturer shall provide the national control authority with adequate data to prove the stability of the product under appropriate conditions of storage and shipping.

5. CONTROL TESTS ON FINAL PRODUCT

5.1 Identity test

An identity test shall be performed on at least one container from each filling lot after reconstituting the vaccine according to the indications of the manufacturer for preparing vaccine for human administration. A high-titre monospecific serum known to be free from neutralizing properties cross-reacting with other group B viruses shall be used. Two different methods may be used for the identity test.

Test in mice. Progressive dilutions in steps not larger than fivefold shall be made of the reconstituted vaccine in diluent for yellow fever virus. Aliquots of each virus dilution shall be mixed with equal volumes of local reference immune serum and similar aliquots mixed with equal volumes of non-immune serum. The serum-virus mixtures shall be incubated at 37°C and each shall be injected into groups of not less than six mice as required in the yellow fever virus neutralization test described in section 3.1.4.2. All mice shall be observed daily for 21 days, and all deaths shall be recorded. Only deaths considered to be caused specifically by yellow fever virus infection shall be taken into account in the computations. Mice paralysed on the twenty-first day shall be counted as alive. The virus dilution calculated to give 50% mortality when mixed with non-immune serum shall be more than tenfold higher than the virus dilution calculated to give 50% mortality when mixed with immune serum.

Test in cell cultures (plaque reduction test). If the test is performed in cell cultures the technique described in section 3.1.4.2 shall be used by mixing dilutions of vaccine with immune and non-immune serum and observing a significant reduction in plaque number.

5.2 Tests for bacteria and fungi

The requirements concerning tests for bacteria and fungi as described in Part A, section 5.2, of the revised Requirements for Biological

Substances No. 6 (revised General Requirements for the Sterility of Biological Substances) [1] shall apply to yellow fever vaccine.

5.3 Tests for other agents

In some countries the national control authority requires that the vaccine should be shown by suitable tests to be free from ALV, mycoplasma or other agents.

If these tests have been done on the bulk product they may be omitted from the final product.

5.4 Innocuity tests

Each filling lot shall be tested for innocuity by appropriate tests in mice and guineapigs using parenteral injections. The test procedures shall be approved by the national control authority.

5.5 Potency test

Three final containers shall be selected at random from each filling lot and shall be tested individually by assay in mice or in cell cultures by a technique shown to be sensitive by assay of a reference preparation of yellow fever vaccine.

Before assay and after reconstitution, in the volume and diluent recommended by the manufacturer for preparing the vaccine for human administration, the vaccine shall stand at a temperature between 20°C and 30°C for 20 minutes before further dilution. This material shall be considered as undiluted vaccine in the calculation of the end-point.

The titre of the vaccine shall be not less than 1000 mouse LD_{50}, or its equivalent in plaque forming units, in the volume of the human dose recommended by the manufacturer for use in man.

Each laboratory choosing to use a cell culture assay shall establish to the satisfaction of the national control authority the relationship between mouse LD_{50} and plaque forming units.

The following techniques have been found satisfactory for the evaluation of the vaccine potency :

Assay in mice

Serial tenfold dilutions of the reconstituted vaccine should be made in diluent for yellow fever virus.

Mice of a highly susceptible strain, 4–6 weeks of age, should be injected intracerebrally under anaesthesia with 0.03 ml of the

[1] WHO Technical Report Series, No. 530, 1973, p. 49.

vaccine dilutions. Groups of not less than six mice should be used for each dilution, and the series of dilutions should cover the range 0–100% mortality of the mice.

Injection of the mice should be performed immediately after the dilutions have been made.

During a period of observation of 21 days, all deaths should be recorded. Only survivors and deaths caused by typical yellow fever infections should be counted in the computations. Mice paralysed on the twenty-first day of observation should be counted as survivors.

Assay in cell cultures

Serial fourfold dilutions of the reconstituted vaccine should be made in Leibovitz medium No. 15 and one of the two techniques outlined below should be used.

Vero cell technique. A cell seed and description of a method for the cultivation of these cells may be obtained through the World Health Organization. Monolayers of cells are prepared in 35 mm Petri dishes, and three dishes should be inoculated with each virus dilution. After infection for 1 hour at 35°C the inoculum (0.2 ml per Petri dish) is replaced by 3 ml of Leibovitz medium pH 7.1–7.2 containing a final concentration of 5% fetal bovine serum and 0.5% agarose. Five days after infection 0.1 ml of the overlay, with the addition of 1 : 50 000 neutral red, is added and plaques are counted on day 6. In calculating the titres all dilutions should be considered in which the average number of plaques per dish is between 1 and 30.

PS cell[1] *technique*. A cell seed and description of a method for the cultivation of these cells may be obtained through the World Health Organization.

The assay technique used is a modification of that of De Madrid & Porterfield.[2] Equal 0.2 ml amounts of a PS cell suspension (approximately 6×10^5 cells/ml) and virus dilution in Leibovitz medium No. 15 (L15)[3] are placed in 16 mm diameter flat-bottomed wells in sterile disposable plastic plates suitable for cell culture. The plates are sealed and incubated for 4 hours at 36°C. After incubation 0.4 ml of overlay medium consisting of L15 medium plus 3% fetal bovine serum and 1.6% of carboxymethylcellulose (low-viscosity sodium salt) is added to each well. The plates should be resealed and incubated at 36°C for 5 days. The plates should then be drained, washed with saline, stained with 1% naphthalene black, rinsed in tap water,

[1] These cells are latently infected with swine fever virus and their importation is prohibited in certain countries.

[2] De Madrid, A. T. & Porterfield, J. S. *Bulletin of the World Health Organization*, 40 : 113–121 (1969).

[3] Leibovitz, A. *American journal of hygiene*, 78 : 173 (1963).

and the plaques counted. All dilutions with a mean number of plaques per well in the range 1–20 are included in the calculation.

5.6 Protein nitrogen content

The protein nitrogen, before the addition of any stabilizer, shall be not more than 0.25 mg per human dose.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply, with the addition of the following.

Written records shall be kept of all seed lots and vaccine lots produced by the manufacturing establishment, irrespective of the results of safety and potency tests.

The format of the records shall be of a type approved by the national control authority.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [2] shall apply with the addition of the following.

In addition to the samples of vaccine lots, samples of all seed lots shall be retained by the manufacturing laboratory and stored under the same conditions as those pertaining to the remainder of the lot until the expiry date of all vaccine lots prepared from these seed lots.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [2] shall apply, with the addition of the following.

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[1] WHO Technical Report Series, No. 323, 1966, p. 17.

[2] WHO Technical Report Series, No. 323, 1966, p. 18.

The label on the package shall include the following additional information :

- the fact that the vaccine fulfils the WHO Requirements ;
- the words " living yellow fever vaccine prepared from chick embryos " ;
- the volume and kind of diluent to be added to reconstitute the vaccine ;
- the volume of the recommended human dose, and the mode of administration (subcutaneous injection) ;
- the words " the dose shall be the same for persons of all ages " ;
- instructions for the administration of the vaccine, including the statement in large bold-face type : " The reconstituted vaccine must be used or discarded within one hour of the opening of the container " ;
- a statement that the vaccine is not recommended for children of less than six months of age ;
- a statement that reconstituted vaccine should be held at a temperature close to 0°C.

9. DISTRIBUTION AND SHIPPING

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply.

10. STORAGE AND EXPIRY DATE

10.1 Storage conditions

Before being distributed by the manufacturing establishment or before being issued from a depot for the maintenance of reserves of vaccine all vaccines shall be kept constantly at a temperature below −20°C.

If possible, storage should be at a temperature of less than −25°C. Since storage below −25°C may prevent deterioration of insufficiently dried products that would deteriorate at higher temperatures, some ampoules should always be stored at 37°C for 2 weeks [2] in order to test the stability of the vaccine.

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[1] WHO Technical Report Series, No. 323, 1966, p. 18.

[2] ROBIN, Y. ET AL. *Bulletin of the World Health Organization*, 44 : 729–737 (1971).

The manufacturer shall recommend such conditions of storage and shipping during distribution as will ensure that the constituted vaccine conforms to the requirements of potency until the expiry date as stated on the label.

> Distributed vaccine should normally be stored at a temperature lower than 4°C although higher temperatures may be permitted for a short interval.
>
> Whatever the temperature of storage and distribution, the vaccine, at its expiry date, should fulfil the requirements for potency as specified in section 5.5, page 39.

10.2 Expiry date

The expiry date shall be not more than 18 months after the date of the last satisfactory potency test, provided that the vaccine has been maintained under the conditions of storage mentioned in section 10.1. The expiry date shall not, however, be more than 12 months from the date on which the vaccine was issued by the manufacturers.

PART B:
NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories given in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply.

2. RELEASE AND CERTIFICATION

Written procedures for the preparation of yellow fever vaccine adopted by the manufacturer must be submitted for approval to the national control authority and to the World Health Organization if the vaccine is to be used to satisfy international requirements for immunization. Proposals for modification must be submitted for approval to the national control authority and to the World Health Organization

[1] WHO Technical Report Series, No. 323, 1966, p. 19.

before their implementation. Protocols of the production and testing processes used in the manufacture of each lot of vaccine must be submitted prior to release to the national control authority as required and also to the World Health Organization. In view of differing local practices in the production of yellow fever vaccine, especially in the blending of single harvests to form a bulk suspension and filling lots, the reporting of data shall be given in three sections, as follows:

(1) data on primary and secondary seed;
(2) data on individual single harvests;
(3) data on filling lots.

The data on each single harvest and filling lot shall be recorded on a separate sheet. The summary protocol (see Appendix) has been designed with this purpose in mind.

The World Health Organization may, on occasion, call for samples of seed lot and final lot containers of yellow fever vaccine for the purpose of testing by independent laboratories.

Before the release of any vaccine from a manufacturing establishment the requirements for consistency of production should be satisfied. This is applicable also to any modification in production method or change of seed lot.

3. SURVEILLANCE FOR ADVERSE REACTIONS

In the case of new manufacturers, change of manufacturing process, or change of seed, the national control authority must ascertain that adequate control of the vaccine has been achieved by arranging for studies in man of some of the lots of vaccine.

Appendix

SUMMARY PROTOCOLS OF YELLOW FEVER VACCINE PRODUCTION

A. Primary/Secondary Seed Lot

Name and address of manufacturing laboratory .....

Laboratory reference number of lot .....

Date when the processing was completed .....

Information on manufacture

1. Virus used to inoculate tissues for the manufacture of the lot:

   (*a*) strain and substrain .....

   (*b*) passage level .....

   (*c*) source and reference number .....

   (*d*) remarks .....

2. Date of inoculation of embryos .....

3. Date of harvesting .....

4. Age of embryos .....

5. Temperature of incubation .....

6. Results of sterility tests .....

7. Number of containers prepared .....

8. Conditions of storage .....

Information on safety tests

1. Monkey safety test

   (*a*) Date of inoculation of monkeys .....

   (*b*) Dilution of the reconstituted seed lot used for the inoculum .....

   (*c*) Virus titre (LD_{50} or PFU) inoculated in each monkey .....

(*d*) Record of monkeys

Monkey	Weight (kg)	Maximum titre of circulating virus (PFU or mouse LD_{50})	Signs of encephalitis and/or paralysis *	Death*	Development of immunity *
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					

* State " yes " or " no "

(*e*) Identity test ..... Date of test .....

2. Sterility test Date of test .....

(*a*) Number of containers examined .....

(*b*) Number showing contamination .....

(*c*) Remarks .....

3. Guineapig safety test Date of test .....

(*a*) Number of animals injected .....

(*b*) Number of animals showing reaction .....

(*c*) Remarks .....

4. Other safety tests .....

5. Remarks .....

Signature of director of manufacturing laboratory ..... Date .....

B. Single Harvest

Name and address of manufacturing laboratory .....

Laboratory reference number of single harvest .....

Information on source materials

1. Virus used to inoculate embryos:

   (*a*) primary seed strain and source .....

   (*b*) passage level of primary seed .....

   (*c*) secondary seed lot, reference number and source .....

2. Embryos used:

   (*a*) is the flock under direct control of manufacturer? .....

   (*b*) is the flock monitored for compliance with the requirements? .....

3. Results of tests on flock or control tissues:

   (*a*) tests for salmonella .....

   (*b*) tests for fowl pox virus .....

   (*c*) tests for avian mycobacteria .....

   (*d*) tests for other microbial agents (give details) .....

Information on manufacture

1. Date of inoculation of embryos .....

2. Date of harvesting .....

3. Age of embryos at harvesting .....

4. Temperature of incubation .....

5. Results of tests of culture for mycobacteria .....

6. Results of sterility test .....

Signature of director of manufacturing laboratory ..... Date .....

C. Filling Lot

1. Name and address of manufacturing laboratory .....

2. Identification of filling lot .....

   Date of preparation .....

3. Identification of single harvest(s) used to prepare filling lot .....

4. Dilution of embryo pulp to make bulk suspension .....

5. Volume of bulk suspension .....

6. Number of ampoules or vials .....

7. Number of human doses per ampoule .....

8. Result of potency test: Date of test .....

   Virus titre per human dose (LD_{50} or PFU)

   1

   2

   3

   Mean geometric titre

9. Result of identity test ..... Date of test .....

10. Result of sterility test: Date of test .....

    (*a*) Number of containers examined .....

    (*b*) Number showing contamination .....

    (*c*) Remarks .....

11. Result of guineapig innocuity test: Date of test _____

(*a*) Number of animals injected _____

(*b*) Number of animals showing reaction _____

(*c*) Remarks _____

12. Result of mouse innocuity test: Date of test _____

(*a*) Number of mice injected _____

(*b*) Number of mice showing reaction _____

(*c*) Remarks _____

13. Protein nitrogen content test Date of test _____

protein nitrogen ____ mg/human dose

14. Other tests _____

_____

15. Remarks _____

_____

Signature of director of
manufacturing laboratory _____ Date _____

Annex 2

REQUIREMENTS FOR MENINGOCOCCAL POLYSACCHARIDE VACCINE

(Requirements for Biological Substances No. 23)

INTRODUCTION

There have been numerous attempts to develop vaccines against meningococcal meningitis employing whole-cell suspensions or autolysates. Unfortunately, however, the data from trials using these vaccines were often difficult to interpret because the trials were not adequately controlled.

Over the last few years considerable progress has been made in the development of vaccines containing purified capsular polysaccharides of Group A and Group C meningococci. Controlled field trials of

the Group A vaccine in Egypt, Finland and the Sudan have demonstrated excellent protection against the clinical disease, and similar studies in the USA with the Group C vaccine have shown it to be 90% effective. The protection is group-specific. As these vaccines are already in use in public health practice in several countries their standardization is necessary.

GENERAL CONSIDERATIONS

Meningococcal polysaccharide vaccines differ substantially from commonly used whole-cell bacterial vaccines and the requirements therefore differ in many particulars from those applied to other vaccines. The most important difference is that these products are made from highly purified polysaccharides that are partially chemically defined so that they can be substantially characterized by their composition and molecular weight.

The evidence suggests that the antibody responses of animals to the purified polysaccharides differ from those observed in man. This factor necessitates an approach different from that applied to the biological standardization of other bacterial vaccines in common use. With meningococcal vaccines it is important that the initial batches from a new manufacturer and even batches prepared by an existing manufacturer using a new process be evaluated in man for immunogenicity by monitoring antibody responses.

Unfortunately the lack of susceptibility of experimental animals to meningococcal infections also makes it impossible to estimate the efficacy of these vaccines for man on the basis of studies in animals. Efficacy can be evaluated at present only by clinical studies. The field trials concluded to date have provided a considerable body of data. However, there is still a lack of definitive data on the efficacy of Group C vaccines in infants and young children. It would therefore be desirable to keep appropriate groups of vaccinated infants and young children under surveillance to obtain such data.

Group A polysaccharide consists of repeating units of *N*,*O*-diacetylmannosamine phosphate linked with 1–6-phosphodiester bonds, and these components, together with calcium ions and residual moisture, account for over 90% of the dry weight of the preparation. Unfortunately this polysaccharide is intrinsically unstable and readily depolymerizes at ambient temperature, but the rate of degradation becomes negligible at temperatures of −20°C or lower. The molecular weight

is thus not readily identifiable, because the molecular size distribution is polydispersed, with components ranging from less than 50 000 to well over 200 000. The distribution of the molecular weight is best estimated by gel filtration on Sepharose 4B, and this procedure has been included as a requirement. It is known that preparations degraded by a prolonged exposure to ambient temperatures are significantly less immunogenic; however, the requirements concerning molecular size are such that they are met relatively easily in production yet allow a margin of safety for changes that may occur under non-ideal but practical conditions.

The Group C polysaccharide is a polymer of *N,O*-diacetylneuraminic acid, and these components, together with calcium ions and residual moisture, account for over 90% of the weight of the material. Unlike Group A polysaccharide this polymer is not susceptible to degradation at ambient temperatures but in all other respects the properties of the two polysaccharides are similar.

Another unusual feature is that purification of the polysaccharides is carried out under chemically clean but not aseptic conditions (because the latter would be prohibitively difficult), the final product being rendered bacteriologically sterile by membrane filtration.

Chemical tests are not carried out on the final bulk but rather on aliquots of lots of purified polysaccharide. If these meet all the requirements they may be pooled to form a final bulk of any desired size. Moreover, any lot of purified polysaccharide that fails to meet any of the chemical requirements may be re-treated and if it is then able to meet the requirements it may be included in a final bulk.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements so that, if a health administration so desires, these parts as they appear may be included in definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance. Detailed descriptions of technical methods that may be used are given in Part C of these requirements.

Should individual countries wish to adopt these requirements as the basis for their national regulations concerning meningococcal vaccine, it is recommended that a clause be included that would permit modifications of manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure a degree of safety and a potency of the vaccine at least equal to those provided by the requirements formulated

below. It is desirable that the World Health Organization should then be informed of the action taken.

The terms "national control authority" and "national control laboratory", as used in these requirements, always refer to the country in which the vaccine is manufactured.

PART A:

MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be *Vaccinum meningitidis cerebrospinalis* followed in parentheses by the serogroup specificity, thus: polysaccharide Group A and/or polysaccharide Group C. The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 Descriptive definition

Vaccinum meningitidis cerebrospinalis shall consist of purified Group A and/or Group C meningococcal polysaccharide(s). The polysaccharides shall be prepared from strains of *Neisseria meningitidis* that have satisfied the requirements of Part A, section 3.1.1, and purified by methods outlined in Part A, sections 3.3.3 and 3.4. The final preparation shall satisfy all the requirements formulated below.

1.3 Terminology

Parent seed lot. A quantity of living *Neisseria meningitidis* organisms of a specific strain processed together and of uniform composition. A parent seed lot may be maintained in the dried form or frozen at temperatures below −45°C.

Working seed lot. A quantity of living *Neisseria meningitidis* organisms derived from the parent seed lot by selecting a colony, growing the organisms and maintaining them in aliquots in the frozen state at −45°C or below.

Single harvest. A sediment obtained on the same day by centrifugation of one culture irrespective of the volume of hexadecyl trimethylammonium bromide[1] that has been added to precipitate the group-specific polysaccharide.

Lot of purified polysaccharide. The material obtained after final purification. The lot may be derived from a single harvest or from several single harvests.

Final bulk. A pool of lots of purified polysaccharide that have been dissolved and filtered through membranes under aseptic conditions. The final bulk is contained in a single vessel from which the final containers are filled.

Filling lot (final lot). A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling or drying. A filling lot must, therefore, have been aseptically filtered, filled, and dried together.

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)[2] shall apply.

Staff assigned to the production of meningococcal vaccine should be vaccinated against the particular serogroup that is under production.

3. PRODUCTION CONTROL

3.1 Control of source of material

3.1.1 *Strains of* Neisseria meningitidis

The strains of *Neisseria meningitidis* Group A and the strains of *N. meningitidis* Group C used for preparing polysaccharide shall be approved by the national licensing authority. They shall also have been shown to be capable of producing polysaccharide known to be safe and effective in man.

The following strains have been shown to be suitable: for Group A polysaccharide—A1, M1027; for Group C polysaccharide—C11, C2241.

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[1] The international nonproprietary name for this substance is cetrimonium bromide.

[2] WHO Technical Report Series, No. 323, 1966, pp. 11-22.

The parent seed lot used shall be identified by a record of its history, including the source from which it was obtained and particulars of all tests made periodically for the verification of strain characters. The cultures shall have the following characteristics: (1) stained smears made from a culture shall be typical of *N. meningitidis*; (2) the organism shall grow at 37°C but not at 25°C; (3) the culture shall ferment glucose and maltose; (4) the colonies on agar shall be oxidase-positive; and (5) a suspension of the culture shall be agglutinated specifically with an appropriate grouping serum.

3.1.2 *Seed lot system*

The production of meningococcal vaccine shall be based on a seed lot system. Cultures of the working seed lot shall have the same characteristics as cultures of the strains from which the parent seed lot was derived (Part A, section 3.1.1). The preparation of seed lots shall be in compliance with the requirements of Part A, section 3.2.

A suitable menstruum for drying or preserving a parent or working seed lot in the frozen state is one containing 5% monosodium glutamate and 5% bovine plasma; in some countries skimmed milk is used. Mueller-Hinton agar medium may be used for the preparation of working seed lots.

3.2 Production precautions

The general production precautions, as formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)[1] shall apply.

Particular efforts are necessary to maintain the culture tanks and all other equipment scrupulously clean. In particular, efforts should be made to avoid cross-contamination of the polysaccharides.

3.2.1 *Culture medium for vaccine production*

The fluid culture medium used for vaccine production shall be free from ingredients that will form a precipitate on addition of hexadecyl trimethylammonium bromide to a final concentration of 1 g/l. It shall be free also from substances that may give rise to sensitization in man if the ingredients appear in the final vaccine.

[1] WHO Technical Report Series, No. 323, 1966, p. 15.

The basal medium described by Frantz [1] supplemented with 2 g/l dialysate of yeast extract may be used. The basal medium may be autoclaved but the supplement, consisting of yeast extract dialysate, magnesium sulfate, and dextrose, should be sterilized by filtration.

3.2.2 *Method of production*

The growth of the cultures shall take place at 35.5°C ± 1.5°C. A working seed lot shall be plated on solid medium and after 12–18 hours of incubation the culture shall be used as an inoculum for liquid precultures. As these precultures reach the stationary phase they shall be used as an inoculum for the production tanks, and the production cultures shall be harvested during the late logarithmic or early stationary phase.

The moment of harvest of the production culture should be determined keeping two considerations in mind. Late harvest tends to improve the final yield of polysaccharide but its average molecular size will be lower. Most often the optimum time for harvest has been 10–12 hours after inoculation.

3.3 Control of production culture

3.3.1 *Freedom from contamination*

Samples of the culture taken before killing shall be tested for bacterial contamination by microscopic examination of Gram-stained smears and by inoculation into appropriate media. Several microscopic fields shall be examined at high magnification such that at least 10 000 organisms have been inspected. If any contaminating bacteria are found the culture or any product derived from it shall be discarded.

3.3.2 *Treatment of production culture*

As soon as freedom from contamination is established by examination of stained smears purification shall be undertaken.

The culture may be harvested directly or after killing the organisms by heating at 56°C for a period of 10 minutes. Two methods exist; either hexadecyl trimethylammonium bromide is added to the culture to a final concentration of 1 g/l, or the culture is first clarified by centrifugation after which hexadecyl trimethylammonium bromide is added to the supernate.

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[1] FRANTZ, I. D. *Journal of bacteriology*, 43 : 757 (1942).

3.3.3 *Purification of polysaccharide*

All steps in the purification procedure shall be carried out in clean glass or plastic ware. Only plastic ware compatible with the solvents in use during the particular step of the procedure shall be used. Reagent grade chemicals or equivalent shall be employed, and aqueous solutions of calcium chloride, sodium acetate, or other salts shall be filtered to remove particulate matter present in these materials. All purification steps shall be carried out in a cold room at a temperature of 8°C ± 5°C with chilled reagents. The precipitated detergent-polysaccharide complex shall be collected as rapidly as possible by centrifugation and, unless preliminary purification is carried out immediately, the sediments shall be stored frozen at −20°C or lower.

3.3.3.1 *Preliminary purification of meningococcal polysaccharide.* The precipitate of hexadecyl trimethylammonium bromide and polysaccharide shall be partially purified, the material resulting from this preliminary purification being referred to as intermediate product. This product shall be stored at −20°C or below. The method of purification shall be approved by the national control authority.

Suitable methods for preliminary purification are shown in the Appendix to these Requirements.

3.3.3.2 *Final purification of meningococcal polysaccharide.* The intermediate product, which contains approximately 50% by weight of the desired polysaccharide (the remainder being principally protein and lipopolysaccharide with endotoxic activity), shall be subjected to further purification. The method of further purification shall be approved by the national control authority.

Suitable methods for further purification are shown in the Appendix to these Requirements.

After final purification the material is referred to as the lot of purified polysaccharide. The final dry product shall be handled with sterile precautions and stored at −20°C or lower.

If tests carried out on the lot of purified meningococcal polysaccharide indicate that nucleic acid, protein, or endotoxic contamination is present, the purification procedure applicable to the particular contaminant may be repeated, followed by the procedure for drying the polysaccharide.

3.4 Chemical requirements for lots of purified polysaccharide

All chemical analyses of all lots shall be based on the dry weight of polysaccharide in the salt form. The moisture content of the purified polysaccharide shall be determined by thermogravimetric analysis at 50°C. In laboratories lacking facilities for thermogravimetric analysis, the moisture content may be determined by the Karl Fischer method or by drying *in vacuo* over P_2O_5 at 37°C until a constant weight is reached. These data shall be used for the calculation of the dry weight.

3.4.1 *Protein content*

Each lot of purified polysaccharide shall contain less than 10 mg of protein per gram of polysaccharide as determined by the method of Lowry et al.[1] using bovine plasma albumin as a reference.

3.4.2 *Nucleic acid content*

Each lot of purified polysaccharide shall contain less than 10 mg of nucleic acid per gram of polysaccharide as determined by spectroscopy, assuming that the internal transmission density of a 10 g/l solution contained in a cell 1 cm wide at 260 nm is 200.

3.4.3 O-*acetyl content*

The *O*-acetyl content of each lot of purified polysaccharide shall be equal to or greater than 2 mmol/g of polysaccharide for Group A and 1.5 mmol/g of polysaccharide for Group C. The *O*-acetyl content shall be determined by the method of Hestrin.[2]

3.4.4 *Phosphorus content*

Each lot of Group A polysaccharide shall contain at least 80 mg/g of phosphorus.

3.4.5 *Sialic acid content*

The sialic acid content of the purified polysaccharide, calculated as free *N*-acetylneuraminic acid (molecular weight 309), shall be not less than 800 mg/g of the dry weight of the isolated product as determined

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[1] LOWRY, O. H. et al. *Journal of biological chemistry*, 193 : 265 (1951).

[2] HESTRIN, S. *Journal of biological chemistry*, 180 : 249 (1949).

by the method of Svennerholm,[1] using the reference sialic acid, *N*-acetylneuraminic acid, as the reference preparation.

3.4.6 *Molecular size*

The molecular size of each lot of purified polysaccharide shall be estimated by gel filtration using Sepharose 4B. Chromatography shall be carried out in a solvent having a concentration of 0.2 ionic strength. The molecular weight shall be determined by measuring the distribution constant K_D of the polysaccharide at the main peak of the elution curve. The K_D value must be no greater than 0.40.[2]

There are clinical data indicating that vaccines with a K_D value as high as 0.45 are immunogenic for man. As there is a general relationship between immunogenicity and molecular weight, however, material with the lower K_D value of 0.40 is considered preferable. Attempts should be made to produce material with still lower K_D values, and when such vaccines are being produced regularly these regulations may be amended to require a reduction in K_D value below the currently specified upper limit of 0.40.

3.5 Preparation of final bulk

The final bulk shall be prepared either from a single lot of purified polysaccharide or from several pooled lots. The polysaccharide shall be dissolved under aseptic conditions in a sterile solution containing mannitol or other suitable materials as the menstruum for freeze-drying and shall be sterilized by membrane filtration. The solvent used shall be free from pyrogenic substances.

Membranes with a pore size of 0.22 µm have been found satisfactory.

3.5.1 *Sterility test on the final bulk*

Each final bulk shall be tested for bacterial sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).[3]

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[1] SVENNERHOLM, L. *Biochimica et biophysica acta*, 24 : 604 (1957).

[2] The technology of meningococcal polysaccharide production is such that it is not easy to obtain agreement on an absolute figure for molecular size. In some countries is has been suggested that the K_D value should be less than or equal to 0.40 and that at least 65% of the material should elute with a K_D value less than or equal to 0.50.

[3] WHO Technical Report Series, No. 530, 1973, pp. 48–52.

3.5.2 *Test for serological specificity*

The final bulk of meningococcal Group A polysaccharide and the final bulk of meningococcal Group C polysaccharide used for the combined vaccine shall be tested for serological specificity and identity by the haemagglutination inhibition assay procedure. The meningococcal Group A final bulk shall specifically inhibit haemagglutination of erythrocytes sensitized with Group A meningococcal antigen in the presence of Group A antibody. There shall be no inhibition of haemagglutination by the vaccine at a minimum final concentration of 100 mg (dry weight) per litre in the presence of meningococcal Group C antiserum and erythrocytes sensitized with Group C meningococcal antigen. The meningococcal Group C final bulk shall specifically inhibit haemagglutination of erythrocytes sensitized with Group C meningococcal antigen in the presence of Group C antibody. There shall be no inhibition of haemagglutination by the vaccine at a minimum final concentration of 100 mg (dry weight) per litre in the presence of meningococcal Group A antiserum and erythrocytes sensitized with Group A meningococcal antigen.

4. FILLING AND DRYING

The requirements concerning filling and containers given in Part A, section 4, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply. The meningococcal vaccine shall be freeze-dried. The Group A and the combined Group A and Group C vaccines shall be stored at a temperature of −20°C or lower whereas the Group C vaccine shall be stored at a temperature of 5°C or lower.

5. CONTROL TESTS ON FINAL PRODUCT

5.1 Identity test

An identity test shall be performed on at least one labelled container from each filling lot. The presence of the group-specific antigen(s) shall be confirmed by tests described in Part A, section 3.5.2.

5.2 Test of contamination by heterologous polysaccharide

The monovalent vaccine shall be tested for the presence of heterologous polysaccharide by the test described in Part A, section 3.5.2.

[1] WHO Technical Report Series, No. 323, 1966, pp. 16–17.

5.3 Concentration of polysaccharide

At least one final container shall be checked to determine that it contains the stated amount of polysaccharide. It shall be shown that Group A vaccine contains 75 mg of phosphorus per gram of polysaccharide and that Group C vaccine contains 750 mg of *N*-acetylneuraminic acid per gram of polysaccharide.[1]

5.4 Sterility tests

Each filling lot shall be tested for sterility according to the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).[2]

5.5 Innocuity tests

5.5.1 *Pyrogenicity test*

Each filling lot shall be tested for pyrogenicity by the intravenous injection of rabbits. Three or more healthy rabbits that have not been injected previously shall be used. The vaccine, reconstituted in the form in which it is to be used, shall be diluted further in pyrogen-free physiological saline so that each rabbit shall receive, by injection into the ear vein, the following doses of dry weight polysaccharide per kilogram of rabbit weight:

Group A vaccine, 0.0025 µg
Group C vaccine, 0.0025 µg
combined Groups A and C vaccine, 0.0050 µg

In each instance the specified dosage level of polysaccharide for each rabbit shall be suspended in 1 ml of physiological saline per kilogram of rabbit weight. The criteria for passing the test shall be those specified in the International Pharmacopoeia.[3]

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[1] The content of phosphorus and *N*-acetylneuraminic acid in the final containers is inevitably somewhat lower than the content of these substances in the vaccine lots, as given in sections 3.4.4 and 3.4.5 of this annex.

[2] WHO Technical Report Series, No. 530, 1973, pp. 48–52.

[3] A detailed description of the test for pyrogens is given in: *Specifications for the quality control of pharmaceutical preparations — second edition of the International Pharmacopoeia.* Geneva, World Health Organization, 1967, Appendix 43, p. 747.

5.5.2 *Tests for abnormal toxicity*

5.5.2.1 *Guineapig toxicity.* No fewer than five guineapigs weighing approximately 350 g each shall be injected intraperitoneally with 500 µg of the polysaccharide(s). The animals shall be observed for 7 days and the injection shall cause neither significant symptoms nor death during this period.

5.5.2.2 *Mouse toxicity.* No fewer than five mice weighing approximately 18 g each shall be injected intraperitoneally with 100 µg of the polysaccharide(s). The animals shall be observed for 7 days and the injection shall cause neither significant symptoms nor death during this period.

5.6 Estimation of molecular size

The molecular size of the polysaccharide in at least one final container from each filling lot shall be determined by Sepharose 4B gel filtration as outlined in Part A, section 3.4.6. The polysaccharide shall elute with a distribution constant, K_D, of 0.40 or less.[1]

5.7 Test for residual moisture

A test shall be performed on at least one container from each filling lot to determine the amount of moisture in the product. The filling lot shall pass the test if the moisture content is not more than 3%. The method used for the determination of the moisture content shall be approved by the national control authority.

5.8 Storage

The Group A vaccine shall be stored at a temperature of −20°C or lower. The Group C vaccine shall be stored at a temperature of 5°C or lower.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories)[2] shall apply.

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[1] See footnote on p. 59.

[2] WHO Technical Report Series, No. 323, 1966, p. 17.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply, with the addition of the following.

The label on the container shall show :

— the number of micrograms of polysaccharide(s) in the human dose

— the volume and nature of the reconstituting fluid.

Furthermore, the label on the container or the label on the carton enclosing several containers, or the leaflet accompanying the container, shall contain the following additional information :

— a statement that after the dried vaccine has been reconstituted it should be used within 8 hours ;

— a statement that the Group A vaccine shall be stored at −20°C or lower ;

— a statement that the Group C vaccine shall be stored at 5°C or lower ;

— a statement that a combined Group A and Group C vaccine shall be stored at −20°C or lower.

9. DISTRIBUTION AND SHIPPING

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply.

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[1] WHO Technical Report Series, No. 323, 1966, p. 18.

10. STORAGE AND EXPIRY DATE

10.1 Storage conditions

The manufacturer shall recommend such conditions of storage and shipping as will ensure that the vaccine conforms to these requirements until the expiry date as stated on the label. The Group A vaccine shall be stored at −20°C or lower; the Group C vaccine shall be stored at 5°C or lower.

10.2 Expiry date

The expiry date for dried vaccines shall be not more than 5 years from the date of harvest or not more than 18 months from the date of issue. The manufacturer shall provide data to confirm the stability of the product.

PART B:

NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply.

In addition, the national control authority shall give directions to manufacturers concerning the most suitable strains from which polysaccharide vaccine shall be produced.

2. RELEASE AND CERTIFICATION

A vaccine lot shall be released only if it fulfils all requirements set forth in Part A of the present document.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify whether the lot of vaccine in question meets all national requirements as well as Part A of the present requirements.

[1] WHO Technical Report Series, No. 323, 1966, pp. 19–22.

The certificate shall also state the date of the last satisfactory test for molecular weight, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of meningococcal polysaccharide vaccine between countries.

3. REACTIVITY AND IMMUNOGENICITY OF VACCINE IN MAN

The national control authorities shall satisfy themselves that adequate control of meningococcal vaccine has been achieved by arranging for studies in man [1] to be made on the first series of production lots and thereafter at regular intervals on some of the vaccine lots. Such studies shall be repeated if any changes in production methods are made.

The national control authorities shall ensure that the studies are done in an adequate number of subjects to obtain statistically valid data on reactivity and immunogenicity.

The following methods of demonstrating safety and efficacy may be used.

Healthy adult human subjects should be immunized in the manner prescribed for the product and their temperatures measured between 4 and 6 hours, and again between 24 and 48 hours, after injection. Such temperatures, as well as all adverse reactions during the first 48-hour period should be reported.

Samples of sera from each subject taken immediately prior to injection and again at 2–4 weeks after immunization should be assayed for bactericidal antibodies. The bactericidal assay should be performed with paired sera from each subject in serial twofold dilutions against the A1 strain for Group A and/or the C-11 strain for Group C or equivalent strains of *Neisseria meningitidis*. The antibody titre should be expressed as the reciprocal of the highest dilution that effects 50% or greater killing of the test organisms. The antibody titres of the sera from at least 90% of the subjects should show a fourfold or greater rise after immunization. If the sera from less than 90% but greater than 80% of the subjects show such a rise, one re-test of the product may be allowed, but in such a case the sera from at least 90% of all subjects in the two tests combined should show a fourfold or greater antibody increase.

[1] The persons should be volunteers.

Appendix

DETAILS OF SOME METHODS AND TESTS USED IN THE MANUFACTURE AND CONTROL OF MENINGOCOCCAL POLYSACCHARIDE VACCINES

These methods and tests are given as a guide to control laboratories.

1. Suitable methods for preliminary purification

A satisfactory method of preliminary purification is as follows. The quantities suggested assume that the sediment from 100 litres of culture is available. The sediment, which may come from a single harvest or may be a pool of several single harvests, is smoothly suspended in 1 litre of distilled water. One litre of 2 mol/l calcium chloride is added to dissociate the detergent-polysaccharide complex and the suspension is stirred for one hour. In order to precipitate the nucleic acids and the bulk of soluble protein, absolute ethanol or 95% ethanol is added to a final ethanol concentration of 250 ml/l. After one hour of standing the precipitate is sedimented by centrifugation at approximately 20 000 *g* for 20 minutes and the perfectly clear supernatant is retained. Unless this solution is free from particulate matter it is probable that the final product will contain an unacceptable level of nucleic acid contamination. Ethanol is added to this solution to a final concentration of 750 ml/l for the Group C polysaccharide and 800 ml/l for the Group A polysaccharide. After agglomeration of the precipitate is complete (usually within one hour), the precipitated polysaccharide is collected by centrifugation at approximately 3000 *g* for 10 minutes. The sediment is washed at least twice with about 1 litre of absolute ethanol (each time) to remove hexadecyl trimethylammonium bromide and calcium chloride. It is essential to use absolute ethanol because unacceptable losses of polysaccharide will occur if 95% ethanol is employed. The sediment is washed twice with approximately 1 litre of acetone and dried in vacuum.

2. Suitable methods for further purification

Two methods for further purification have been used. The first is based on the method of Sevag [1] and consists of homogenizing in a blender an aqueous solution of the intermediate product (about 20 g/l) and a mixture of chloroform and *n*-butanol (in the ratio of 5 : 1). After centrifugation for 10 minutes at approximately 10 000 *g*, the clear aqueous supernatant is decanted and the homogenization of this layer repeated until a negligible interfacial precipitate forms on centrifugation.

The other method depends on cold-phenol extraction. The intermediate product is dissolved in 0.1 saturated neutral sodium acetate to give a concentration of intermediate product of 20 g/l. It is then extracted three times with twice its volume of cold phenol (100 g crystalline phenol dissolved in 40 ml of 0.1 saturated neutral sodium acetate). The extraction consists of vigorous shaking by hand for approximately 30 seconds. To minimize losses the three phenol layers are sequentially extracted with twice their volume of distilled water and added to the first aqueous supernatant. Phase separation is achieved by centrifugation for 15 minutes at 35 000 *g*. The polysaccharide solution is dialysed for 24 hours against cold 0.1 mol/l calcium chloride.

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[1] SEVAG, M. G. *Biochemische Zeitschrift*, 273 : 419 (1934).

The solution is centrifuged for 3 hours at 100 000 *g* to sediment endotoxic lipopolysaccharide. Ethanol is added to the supernatant to a final concentration of 750 ml/l. The precipitated polysaccharide is collected by centrifugation, washed twice with absolute ethanol and twice with acetone. It is then dried in vacuum.

For the production of the Group A polysaccharide the cold-phenol procedure is to be preferred because the yield and molecular size are much higher. This procedure can also be used for the production of Group C polysaccharide, but frequently endotoxic activity is not readily removed by ultra-centrifugation unless one or more cycles of homogenization with a mixture of chloroform and butanol have been carried out.

3. Molecular sizing of capsular polysaccharide antigens by Sepharose 4B column chromatography

Reagents[1]

Sepharose 4B gel
0.2 mol/l ammonium acetate, pH 7.0, as eluant
^{14}C-labelled sodium acetate
Blue dextran 2000
Scintillation liquid
Reagents for chemical assay of polysaccharides

Equipment

Column (1.5×90 cm)
Automatic fraction collector and test-tubes
Gel and eluant reservoir
Liquid scintillation counter and counting vials
Refractometer or other appropriate monitoring device and recorder (optional)

Procedures

(1) Washing of the Sepharose 4B gel:

(*a*) To approximately 200 ml of packed Sepharose 4B gel add 400 ml of 0.2 mol/l ammonium acetate. Stir the gel thoroughly and let it sediment for about 1 hour. Remove the supernatant fluid together with the fine particles suspended in it.

(*b*) Repeat the above procedure until there are no fine particles in the supernatant.

(*c*) Decant all the supernatant and add 200 ml of fresh 0.2 mol/l ammonium acetate to the gel. De-aerate the gel suspension in a vacuum.

(2) Packing of the column:

(*a*) Mount the column vertically and connect it to the gel and eluant reservoir. Fill the column with 0.2 mol/l ammonium acetate to a depth of about 30 cm.

(*b*) Pour the washed Sepharose 4B slurry into the reservoir slowly to avoid trapping air bubbles and adjust the height of the surface of the slurry above

[1] Advice on the availability of suitable products may be obtained from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

the column outlet to about 70–75 cm. Open the column outlet and let the gel sediment at that operating pressure for about 16 hours ; the flow rate of the column is approximately 15–20 ml/h.

(*c*) When the column is packed to a height of about 87 cm, equilibrate the gel bed with two to three column volumes of eluant. The operating pressure should always be equal to or less than that at which the column was packed. Mark the gel bed level.

(3) Calibration of the column :

(*a*) Void volume (V_0) :

(i) The void volume is determined with blue dextran dissolved in 0.2 mol/l ammonium acetate to a concentration of 2 g/l.

(ii) Drain the excess eluant from the column until the eluant level is equal to the gel bed level ; close the column outlet.

(iii) Carefully layer 1 ml of blue dextran on top of the gel bed and allow the sample to drain into the gel bed by opening the column outlet.

(iv) When the sample level is equal to the gel bed level, fill the column with eluant and connect it to the eluant reservoir. Start collecting the fractions (approximately 2 ml per fraction) with an automatic fractionator. (Simultaneously, the eluant may be monitored for blue dextran with a monitoring device and a recorder.)

(v) The internal transmission density (optical density) of each fraction is read at 260 nm and plotted against the number of fractions. V_0 is determined as the volume of eluant collected up to the position of the maximum of the first blue dextran peak in the elution diagram. Determination of the position of the apex of the elution peak may be done by extending upwards the two straight lines on the slopes. The interception of the two slopes is taken as the apex of the elution peak.

(*b*) Total bed volume (V_t) :

(i) The total bed volume is determined with ^{14}C-labelled sodium acetate. The column is loaded with 1 ml of the sodium acetate solution containing 0.4 µCi of radioactivity.

(ii) Fractions of about 2 ml each are collected and a 0.5 ml portion from each fraction is transferred into separate scintillation vials containing 10 ml of scintillation liquid. The vials are counted in a liquid scintillation counter.

(iii) The radioactivity, expressed as counts per minute, is plotted against the respective fraction numbers. The total bed volume is the volume of eluant collected up to the position of the maximum of the ^{14}C sodium acetate peak in the elution diagram. Symmetrical peaks in the elution profiles are indicative of the acceptability of the column.

(4) Vaccine measurement :

(*a*) Preparations of meningococcal polysaccharide vaccine Group A or Group C or Groups A and C combined in the final containers are reconstituted with water to a concentration of 2.5 g/l of antigen for Group A or Group C or

5.0 g/l of antigens for Groups A and C combined. The vaccine pool should contain at least 2.2 ml. No more than 10 mg of polysaccharide should be used.

(*b*) One millilitre of the vaccine is used to charge the column, the column is eluted with a volume of 0.2 mol/l ammonium acetate using not less than 1.5 times the total bed volume of the column, and the eluate is collected.

(*c*) The quantity of phosphorus in fractions of Group A vaccine and the quantity of sialic acid in fractions of Group C vaccine are determined. The readings are plotted against the number of fractions. Alternatively, the eluate may be monitored with a refractometer or an absorptiometer and a recorder. However, the profile thus obtained by the monitoring device should be compared with a profile of the same type of vaccine as determined by chemical assay of fractions eluted under identical chromatographic conditions. Immunochemical analyses of the polysaccharides are not sufficiently quantitative to be reliable.

(*d*) The elution volume V_e is measured at the main peak of the polysaccharide elution curve, and the distribution constant is calculated from the equation :

$$K_D = \frac{V_e - V_0}{V_t - V_0}$$

using the values of V_0 and V_t determined for the column. The K_D value of the polysaccharide must be no greater than 0.40.[1]

4. Determination of the *O*-acetyl group in meningococcal polysaccharides Group A and Group C

The methods described in this section are based on the work of Hestrin[2] and Kabat & Mayer.[3]

Instrumentation

Spectrophotometer for the ultraviolet and visible regions of the spectrum.

Mechanism

O-acetyl groups react with hydroxylamine in alkali to form hydroxamic acid. The hydroxamic acid formed is measured by the formation of a coloured complex with Fe^{3+} in acid solution.

Reagents

(1) 2 mol/l hydroxylamine hydrochloride. Store in the cold.
(2) 3.5 mol/l NaOH.
(3) Concentrated HCl (specific gravity 1.18), diluted with twice its volume of water.
(4) 0.37 mol/l solution of $FeCl_3.6H_2O$ in 0.1 mol/l HCl.
(5) 0.001 mol/l sodium acetate, pH 4.5.
(6) Reference solution of acetylcholine chloride (molecular weight 181.7).

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[1] See footnote on p. 59.

[2] HESTRIN, S. *Journal of biological chemistry*, **180** : 249 (1949).

[3] KABAT, E. & MAYER, M. *Experimental immunochemistry*. Springfield, Charles C. Thomas, 1971, pp. 493–494.

Method of preparing reference solution

The reference solution should contain 150 mg acetylcholine chloride per vial. To each vial 10 ml of 0.001 mol/l sodium acetate (pH 4.5) are added with a volumetric pipette. One millilitre of this solution is transferred to a test tube and mixed with 4 ml of 0.001 mol/l sodium acetate (pH 4.5), using a 5 ml volumetric flask if available. From this solution 0.1, 0.2, 0.3, 0.4, and 0.5 ml quantities are transferred accurately in duplicate to 10 test tubes, and 0.9, 0.8, 0.7, 0.6 and 0.5 ml of water are added to make the volume in each tube 1 ml. These reference tubes contain respectively 1.66, 3.32, 5.0, 6.64 and 8.30 micromoles of *O*-acetyl per millilitre. The duplicate at each concentration is used as a reference blank. The approximate internal transmission densities of these solutions at 540 nm in the visible range of the spectrophotometer are 0.2, 0.5, 0.8, 1.0 and 1.3. The readings are not taken at the maximum of absorption but rather on the shoulder of the peak. Further details are given by Hestrin.[1] The reaction is carried out at room temperature.

A reference blank is run for each standard prepared as indicated above. Acid is added to the blanks before the NaOH-hydroxylamine solution is added.

Procedure

(1) Prepare the sample for analysis by making up a 0.1% solution of the polysaccharide (no salt) in water. For each test use 1 ml of this solution, which contains 1 mg of polysaccharide.

(2) To 1 ml of the sample and reference preparations add 2 ml of a freshly prepared (within 3 hours) mixture of equal parts of reagents (1) and (2) (2 mol/l hydroxylamine, and 3.5 mol/l sodium hydroxide) and mix.

(3) After 4 minutes at room temperature, add 1 ml of reagent (3), HCl, to bring the pH to about 1.2 ± 0.2, and mix.

(4) Add 1 ml of reagent (4), ferric chloride, and mix.

(5) Swirl the contents of the test tubes rapidly during the addition of each of the above reagents.

(6) Both a reference blank (one blank for each concentration of the reference) and a sample blank (1 ml of 0.1% polysaccharide solution) must be included. In Hestrin's method the blank determination is called the test for non-specific colour. Acid is added to the blanks before the NaOH-hydroxylamine solution is added.

(7) Read the internal transmission density of the purple-brown colour at 540 nm promptly.

(8) Substract the sample blank readings from those of the samples and subtract the reference blank readings from those of the reference preparations.

(9) Plot a curve of internal transmission density versus molarity of *O*-acetyl. The values of internal transmission density of the samples on the reference curve correspond to micromoles of *O*-acetyl per milligram of polysaccharide (since the test was conducted on 1 ml of the polysaccharide solution, which contains 1 mg of polysaccharide).

The weight of polysaccharide used in the 1 g/l polysaccharide solution is corrected for its moisture content[2] and the final result is expressed as "millimoles of *O*-acetyl per gram of dry polysaccharide".

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[1] HESTRIN, S. *Journal of biological chemistry*, 180 : 249 (1949).

[2] See section 3.4 of Annex 2.

Example : Moisture content = 10%. Thus 1 mg of " wet " polysaccharide comprises 0.1 mg of water and 0.9 mg of " dry " polysaccharide. If, for every milligram of " wet " polysaccharide there are 1.95 µmol of *O*-acetyl, this figure is equivalent to :

$$\frac{1.95\ \mu\text{mol } O\text{-acetyl}}{1\ \text{mg "wet" polysaccharide}}$$

$$= \frac{1.95\ \mu\text{mol } O\text{-acetyl}}{0.9\ \text{mg " dry " polysaccharide}}$$

$$= 2.16\ \mu\text{mol } O\text{-acetyl per milligram of " dry " polysaccharide.}$$

5. Procedures for haemagglutination-inhibition assay for meningococcal polysaccharide vaccine

Sensitization of erythrocytes

Sensitized erythrocytes are prepared according to the method of Artenstein et al.[1] with the following modifications :

(1) Dulbecco's phosphate-buffered saline, pH 7.4, with calcium and magnesium salts is used not only as a diluent but for washing cells as well.

(2) Sheep erythrocytes (washed three times) are used.

(3) Antisera are heated at 56°C for 30 minutes and adsorbed on to packed normal sheep erythrocytes before use.

(4) Sensitizing antigens (Group A or Group C polysaccharides) are used at a concentration of 20 mg/l.

Titration of antisera

Sera are titrated in triplicate using microtitre U-plates.

(1) 0.025 ml of buffered saline is added to each well.

(2) 0.025 ml of antiserum is added to the first well of each row and dilutions are made with 0.025 ml loops down the rows of the wells.

(3) 0.025 ml of buffer is added to each well after the antiserum has been diluted.

(4) 0.05 ml of sensitized cells is added to each well. The plates are incubated for 1–2 hours at room temperature for Group C antigen-coated cells and at 4°C for Group A antigen-coated cells. The cell control consists of 0.05 ml of sensitized cells and 0.05 ml of normal serum or saline.

The highest serum dilution giving a definitive haemagglutination pattern is considered as having one unit of activity. The preceding well is considered to have two units of activity and the next preceding well four units, etc.

A titration of antiserum over an appropriate range of dilutions is included with each haemagglutination inhibition test.

Haemagglutination inhibition

(1) The test vaccine to be used as inhibitor is reconstituted with diluent so that its final concentration in the first well will be at least 100 mg/l. It should be noted that, since the dilution factor for the test vaccine in the first well is 1 : 8, the initial vaccine concentration should be at least 800 mg/l.

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[1] ARTENSTEIN, M. S. et al. *Journal of infectious diseases*, **124** : 277–288 (1971).

(2) 0.025 ml diluent is added to each well.

(3) 0.025 ml test vaccine is added to the first well of each row. Using 0.025 ml loops, make serial twofold dilutions.

(4) The antiserum titrated as described above (see section on titration of antisera) is diluted in tubes to cover a range of at least 1, 2, 4, and 8 units. A 0.025 ml quantity of each antiserum dilution is added to each well of the appropriate rows of vaccine dilutions.

The microtitre plate is gently swirled to ensure the mixing of the material in each well and is incubated at 37°C for 30 minutes.

(5) 0.05 ml of the appropriate sensitized cells is added to each well.

The cell control consists of two drops (0.05 ml total) of diluent in place of the test vaccine and antiserum. A known inhibitor should be included in each experiment as antigen control. Incubation is continued for 1–2 hours at room temperature for Group C antigen-coated cells and at 4°C for Group A antigen-coated cells.

A positive test for inhibition is defined as a reduction of the titre of the titrated antiserum by two twofold dilution steps or more by any vaccine dilution with a final concentration in the well of 100 mg/l or less.

6. Bactericidal antibody assay

Materials [1]

Tissue culture trays, 20 × 30 cm, 96 cups, 2 ml per cup, with sterile covers
Sterile automatic syringes, 1 and 2 ml
Disposable serological pipettes, 1 ml, sterile glass plugged
Nephelometer flasks with side-arm, 300 ml capacity
Nephelometer
Sterile pipettes and test-tubes
Liquid medium: Mueller-Hinton broth or soybean-casein digest broth
Solid medium: soybean-casein digest broth with 1.2% agarose
Normal horse serum, heat-inactivated
Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium
0.85% saline, sterile
Complement from baby rabbits (3–4 weeks old), or freshly collected baby rabbit (approximately 3 weeks old) serum stored at −80°C. Complement should be pre-screened for the absence of anti-meningococcal activity.

Procedure

(1) Serum samples:

(*a*) All test sera are diluted 1 : 2 in DPBS and inactivated at 56°C for 30 minutes. A reference is included as positive control.

(*b*) Serial twofold dilutions of antisera are made by placing 0.2 ml DPBS in each cup of the tray, using a sterile 1-ml automatic syringe, and adding

[1] Advice on the availability of suitable products may be obtained from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

0.2 ml of inactivated test serum to the first well, using a sterile disposable 1-ml pipette aspirated by a 1-ml automatic syringe connected to it by a piece of rubber tubing (taking care not to fill the syringe). The serum is mixed, and 0.2 ml of the mixed serum is transferred to the next well using the same pipette. This dilution process is continued successively through the rest of the wells, the 0.2 ml taken from the last well being discarded.

(2) Bacteria :

(*a*) A lyophilized meningococcal culture or a recently transferred culture is inoculated on to the solid medium and incubated overnight at 37°C in air containing 5% CO_2. A 25-ml quantity of broth is added aseptically to a nephelometer flask and a 5-ml quantity to a sterile culture tube. Approximately $\frac{1}{4}$ loopful of the overnight culture is transferred to the 5 ml of broth in the culture tube. After mixing thoroughly, the culture is poured into the flask and incubated at 37°C on a shaker for approximately 5 hours. Turbidity is read at 530 nm and the number of cells (colony forming units) per millilitre is determined from a standard curve. The culture is diluted in saline to a concentration of 500 cells/ml.

(*b*) 0.2 ml of the cell suspension is added to each well, using a sterile 1-ml automatic syringe.

(3) Complement :

(*a*) 0.2 ml of pre-screened complement is added to each well with a 1-ml automatic syringe.

(*b*) The following controls are included.

(i) Cell control : 0.2 ml DPBS, 0.1 ml bacteria, and 0.1 ml heat-inactivated complement.

(ii) Complement control : 0.2 ml DPBS, 0.1 ml bacteria, and 0.1 ml of active complement.

(iii) Serum control : 0.2 ml serum, 0.1 ml bacteria, 0.1 ml DPBS.

(4) Incubation :

(*a*) The plates are covered and incubated at 37°C for 30 minutes in 5% CO_2.
(*b*) Sterile solid medium is prepared afresh each time in the following manner. Agar gel is heated to 120°C and sterilized in an autoclave. When it has cooled to approximately 45°C, normal horse serum (heat-inactivated) is added to a concentration of 10 ml/l. One millilitre of the medium is added to each well, using a warm 2-ml automatic syringe. The trays are then gently swirled and the medium is allowed to set at room temperature. The trays are covered and incubated at 37°C for 18–24 hours in 5% CO_2.

(5) Reading of results :

The growth in the complement control is used as growth control, which should be the same or only slightly less than that of the cell control. The end-point of each serum titration is the last well that gives 50 % or greater killing as compared with the cell control. Some sera, especially high-titred samples, may have a prozone. Sera showing a killing effect in the serum control should be titrated without complement to determine the extent of this killing effect.

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[1] WHO Technical Report Series, No. 588, 1976.

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Annex 3

SPECIFICATIONS OF TESTS USED IN THE REQUIREMENTS FOR *BRUCELLA ABORTUS* STRAIN 19 VACCINE (LIVE – FOR VETERINARY USE)

(Requirements for Biological Substances No. 20) [1]

Addendum 1975 [2]

The tests in this document merely give the details of the tests to be applied in the Requirements for *Brucella abortus* Strain 19 Vaccine (Live—for Veterinary Use). The tests for which clarification has been given are the following.

1. *Tests applied to seed lots only*

Conventional methods

- CO_2 requirement
- Growth in the presence of dyes
- Production of hydrogen sulfide
- Tests with monospecific antisera

Definitive methods

- Susceptibility to phage
- Growth in the presence of thionin blue
- Growth in the presence of penicillin
- Growth in the presence of erythritol
- Oxidative metabolic tests

2. *Tests applied both to seed lots and to finished vaccine*

Reactivity and antigenicity test in guineapigs

Antigenicity test in calves

Immunogenicity test in guineapigs

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[1] WHO Technical Report Series, No. 444, 1970, pp. 58–70.

[2] Prepared by the Central Veterinary Laboratory, Weybridge, England, and the US Department of Hygiene, National Animal Disease Department, Ames, Iowa, USA.

1. Tests Applied to Seed Lots Only

All the conventional methods and the first four definitive methods specified below are described by Alton et al.[1] The oxidative metabolic tests (pp. 78–79) are described by Meyer & Cameron[2] and Morgan & Gower.[3]

Conventional methods

CO_2 requirement

Strain 19, in common with certain biotypes of naturally occurring *B. abortus* and with certain other species including *B. melitensis* and *B. suis*, does not require added CO_2 for growth.

Growth in the presence of dyes

Strain 19 of *B. abortus* biotype 1 has the characteristics shown in the following table.

Dilution of dye	Degree of inhibition by	
	thionin	basic fuchsin
1 : 25 000	Complete	Partial to complete
1 : 50 000	Complete	Partial to none
1 : 100 000	Complete	None

Production of hydrogen sulfide

Strain 19 is typical of *B. abortus* biotype 1, producing a moderate amount of hydrogen sulfide.

Tests with monospecific B. abortus *and* B. melitensis *antisera*

Strain 19 is typical of *B. abortus* biotype 1, being agglutinated by *B. abortus* monospecific antisera and not by *B. melitensis* monospecific antisera.

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[1] ALTON, G. G. ET AL. *Laboratory techniques in brucellosis*, 2nd ed. Geneva, World Health Organization, 1975 (Monograph Series, No. 55).

[2] MEYER, M. E. & CAMERON, H. S. *Journal of bacteriology*, **82**: 387, 396 (1961).

[3] MORGAN, W. J. B. & GOWER, S. G. M. Techniques in the identification and classification of *Brucella*. In: Gibbs, B. M. & Skinner, F. A., ed. *Identification methods for microbiologists, Part A*. London, Academic Press, 1966, pp. 35–50.

Definitive methods

Susceptibility to Brucella *bacteriophage Tbilisi (Tb)*

All strains of *B. abortus* in the smooth or smooth-intermediate phase are lysed by Tb phage at the routine test dilution. Rough and other non-smooth phases are not lysed.

Growth in the presence of thionin blue

The growth of strain 19, *B. abortus* biotype 2, and an occasional strain of other biotypes is inhibited in the presence of thionin blue at a final concentration of 1 : 500 000.

Growth in the presence of penicillin

The growth of strain 19, *B. abortus* biotype 2, and an occasional strain of other biotypes is inhibited in the presence of penicillin at a concentration of 5 units per millilitre of medium.

Growth in the presence of erythritol

The growth of strain 19 is inhibited in the presence of erythritol at a concentration of 1 g of erythritol per litre of medium.

Oxidative metabolic tests

The oxidative metabolic pattern of strain 19 on substrates recommended for species identity is typical of *B. abortus*; however, there are consistent quantitative differences between strain 19 and other strains of *B. abortus* in the oxidative rates on certain substrates, which are as follows:

(1) The oxidative rate of strain 19 on L-glutamate is consistently higher than that of other strains of *B. abortus*.

(2) The oxidative rate of strain 19 on *i*-erythritol is consistently lower than that of other strains of *B. abortus*.

(3) The oxidative rate of strain 19 on L-alanine is approximately double the oxidative rate on D-alanine.

(4) The oxidative rate of strain 19 on (+)galactose is approximately equal to the oxidative rate on D-ribose.

These points should be considered collectively as representative of strain 19, since other strains of *B. abortus* may exhibit one or other of these characteristics.

The following oxidative rates were obtained on 30 serial lots of strain 19 seed cultures produced between 1956 and 1968 at the National Animal Disease Laboratory (Diagnostic Services), Ames, Iowa, USA.

Substrate	Oxidative rates QO_2N	
	mean	range
D-alanine[a]	92	60–121
L-alanine	179	99–239
L-asparagine	206	154–192
L-glutamate	604[b]	507–690[b]
D, L-ornithine	89	30–124
D, L-citrulline	40	21–59
L-lysine	30	6–48
L-arginine	84	37–119
L-arabinose	132	55–198
(+)galactose	244	176–305
D-ribose	250	210–316
i-erythritol	23	10–35

[a] D-alanine is not used routinely by many laboratories.
[b] Some workers have found lower values for L-glutamate.

2. Tests Applied Both to Seed Lots and to Finished Vaccine

Reactivity and antigenicity tests in guineapigs

Some control authorities require test A. Test B is an alternative procedure that may be demanded by other control authorities.

Test A

Strain 19 vaccine is appropriately reconstituted and inoculated onto slants of a suitable medium, such as potato infusion agar, tryptose agar, or glycerol-dextrose agar, and incubated at 37°C for 48 hours. The growth is washed from the slants with phosphate buffered saline, pH 6.4, and the concentration of cells adjusted to approximately $2–3 \times 10^9$ per millilitre by performing a viable count.

Twelve male guineapigs, preferably weighing approximately 600 g each, are weighed and each is inoculated subcutaneously in the inguinal

area with 1 ml of the standardized suspension of strain 19. Six similar uninoculated guineapigs are kept as controls and are also weighed. After approximately 35 days, the guineapigs are weighed, a blood sample is taken from each, and necropsies are carried out. The internal organs, especially the spleen, are examined for macroscopic lesions. The spleen of each animal is removed aseptically, making sure to remove all fat and connective tissue, and then weighed and cultured.

The mean spleen/body-weight ratio of the control and strain-19-inoculated groups of animals should be comparable and should be approximately 0.15–0.16%.

Small nodular lesions are usually observed in the spleens of one third or fewer of the strain-19-inoculated animals. No significant lesions are observed in other organs or in the control animals.

Measure a volume of physiological saline or 10 g/l peptone solution equal to double (in millilitres) the spleen weight (in grams) into a sterile tissue grinder. Aseptically add the spleen and grind thoroughly. Make a tenfold dilution of the splenic suspension by adding 1 ml of suspension to 9 ml of sterile saline or 10 g/l peptone. Repeat procedure to make hundredfold and thousandfold dilutions of the splenic suspension. Disposable 1 ml pipettes, preferably having large tip openings that are not easily plugged by the splenic pulp, may be used to facilitate this procedure. Deliver 0.1 ml of the tenfold and thousandfold dilutions on each of two tryptose agar plates per dilution. Spread with a sterile spreader. Incubate at 37°C for 4 days. Count the colonies and calculate a single mean for both dilutions. Calculate the total spleen count from the dilution factors and the original volume of diluent used in grinding the spleen, disregarding the volume contributed by the splenic pulp. The mean of the spleen counts of the 12 guineapigs should be approximately 5×10^3 and should not exceed 10×10^3. Strain 19 is recovered from the spleens of 50–100% of the guineapigs.

A serum sample from each animal is tested by the tube agglutination test. An average titre of between 100 and 200 International Units per millilitre of serum should be obtained for the strain-19-inoculated animals. The control animals should be negative.

Test B

Not less than two, and preferably 10, healthy guineapigs, each weighing 250–400 g, are each injected intramuscularly with one-fifteenth of the calf dose of vaccine in a volume of 1 ml and observed for 10 days; no abnormal reaction should develop. The guineapigs are killed on the eleventh day after injection, the blood is collected, and the spleen is

removed, weighed, ground into a suspension in a sterile container and dilutions ranging from 1 : 5 to 1 : 5000 prepared in suitable diluent. Two plates of suitable medium are seeded with 0.2 ml of spleen suspension at each dilution and incubated at 37°C for 4 days. The plates are examined for the presence of *B. abortus* and the colonies counted. The serum from each animal should contain not more than 1000 International Units per millilitre, and the spleen of each animal should contain not more than 500 000 *B. abortus* organisms in 1 g of fresh tissue.

Antigenicity test in calves

This procedure is performed as an additional test if any change is made in the production method.

At least 10 healthy calves which have not been vaccinated against brucellosis are chosen. The ages of the calves should preferably span the age range during which vaccination is permitted or recommended. A blood sample is taken from each calf and each is inoculated subcutaneously with the field dose of the vaccine. The calves are observed for at least 3 days. During this time they usually develop a febrile response and local reaction, but these should quickly resolve themselves. A second blood sample is taken from each calf 21–35 days after vaccination.

Serum from the blood samples is tested by the serum agglutination test for brucellosis. Calves reach their maximum agglutinin response at about 12–15 days after vaccination. At the second bleeding their sera should contain between 40 and 400 International Units per millilitre. The initial samples should be negative.

Immunogenicity test in guineapigs

No fewer than 12 healthy guineapigs, each weighing 300–450 g, are each injected intramuscularly with one-fifteenth of the calf dose of vaccine in a volume of 1 ml. Female guineapigs are to be preferred since they are more readily infected with *B. abortus*. After an interval of 6–8 weeks, the vaccinated guineapigs are inoculated intramuscularly with 1 ml of a suspension containing 5000 (± 20%) *B. abortus* organisms derived from a virulent CO_2-dependent strain of known characteristics; no fewer than six similar unvaccinated guineapigs are similarly injected at the same time. After a further interval of 5–6 weeks, the guineapigs are killed and their spleens are weighed and emulsified separately, a separate suspension of each spleen being prepared in a suitable diluent.

A volume of each suspension, equivalent to 0.05 g of the spleen, is inoculated onto a suitable medium and incubated at 37°C for 4 days in an atmosphere containing 10% CO_2. If colonies of *Brucella* organisms develop, they are tested for CO_2 dependence. If *B. abortus* organisms of the challenge strain are recovered from more than 25% of the spleens, the vaccine fails the test. For the test to be valid, the challenge strain must be recovered from the spleens of all the control guineapigs.

The challenge strain used in this test must be fully virulent. A suitable strain can be obtained from the Central Veterinary Laboratory, Weybridge, United Kingdom. A stock of the challenge strain should be kept in the dried form but the suspension of organisms used in the test should be prepared from an actively growing culture.

Each new dried stock should be subjected to the following two tests for virulence in unvaccinated guineapigs.

(1) The ID_{50} (i.e., the dose needed to infect 50% of the guineapigs) should not exceed 200 organisms.

(2) 10 000 organisms are inoculated into each of a group of 10 guineapigs and the animals are killed 6 weeks later. The geometric-mean spleen/body-weight ratio should not be less than 0.3%.

From time to time it is desirable to perform a three-point immunogenicity test on the vaccine. This is more sensitive than the one-point test and should be used as a critical test of any new production procedure. The test consists in comparing the ID_{50} of the challenge strain in vaccinated guineapigs with that in unvaccinated guineapigs. Three groups of vaccinated guineapigs and three of unvaccinated guineapigs, each group consisting of at least 10 animals, may be used. The guineapigs in each group are given graded doses of the challenge strain. The doses are determined by pilot experiments, a range for the three groups being chosen to span the 50% infection level. From the results an ID_{50} evaluation for the vaccinated and control animals may be obtained, and the ratio of the two evaluations should be approximately 200–2000.

Annex 4

REQUIREMENTS FOR BIOLOGICAL SUBSTANCES AND OTHER SETS OF RECOMMENDATIONS

The specification of requirements to be fulfilled by preparations of biological substances is necessary in order to ensure that these products are safe, reliable, and potent prophylactic or therapeutic agents. International recommendations on requirements are intended to facilitate the exchange of biological substances between different countries and to provide guidance to workers responsible for the production of these substances as well as to others who may have to decide upon appropriate methods of assay and control.

Recommended requirements and sets of recommendations concerned with biological substances formulated by international groups of experts and published in the WHO Technical Report Series are listed hereunder :

No.	Year	
178	1959	Requirements for Biological Substances :
		1. General Requirements for Manufacturing Establishments and Control Laboratories
		2. Requirements for Poliomyelitis Vaccine (Inactivated)
179	1959	Requirements for Biological Substances :
		3. Requirements for Yellow Fever Vaccine
		4. Requirements for Cholera Vaccine
180	1959	Requirements for Biological Substances :
		5. Requirements for Smallpox Vaccine
200	1960	Requirements for Biological Substances :
		6. General Requirements for the Sterility of Biological Substances
237	1962	Requirements for Biological Substances :
		7. Requirements for Poliomyelitis Vaccine (Oral)
274	1964	WHO Expert Committee on Biological Standardization :
		8. Requirements for Pertussis Vaccine
		9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate

293 1964 WHO Expert Committee on Biological Standardization :

10. Requirements for Diphtheria Toxoid and Tetanus Toxoid

323 1966 WHO Expert Group :

Requirements for Biological Substances (Revised 1965)

1. General Requirements for Manufacturing Establishments and Control Laboratories
2. Requirements for Poliomyelitis Vaccine (Inactivated)
7. Requirements for Poliomyelitis Vaccine (Oral)
5. Requirements for Smallpox Vaccine

329 1966 WHO Expert Committee on Biological Standardization :

11. Requirements for Dried BCG Vaccine
12. Requirements for Measles Vaccine (Live) and Measles Vaccine (Inactivated)

361 1967 WHO Expert Committee on Biological Standardization :

13. Requirements for Anthrax Spore Vaccine (Live—for Veterinary Use)
14. Requirements for Human Immunoglobulin
15. Requirements for Typhoid Vaccine
9. Requirements of Procaine Benzylpenicillin in Oil with Aluminium Monostearate (Revisions adopted 1966)

384 1968 WHO Expert Committee on Biological Standardization :

16. Requirements for Tuberculins
17. Requirements for Inactivated Influenza Vaccine

413 1969 WHO Expert Committee on Biological Standardization :

4. Requirements for Cholera Vaccine (Revised 1968)
18. Requirements for Immune Sera of Animal Origin

444 1970 WHO Expert Committee on Biological Standardization :

19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)
20. Requirements for *Brucella abortus* Strain 19 Vaccine (Live—for Veterinary Use)

444 1970 WHO Expert Committee on Biological Standardization :

Development of a National Control Laboratory for Biological Substances (A guide to the provision of technical facilities)

463 1971 WHO Expert Committee on Biological Standardization :

21. Requirements for Snake Antivenins

486 1972 WHO Expert Committee on Biological Standardization :

7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971)

530 1973 WHO Expert Committee on Biological Standardization :

4. Requirements for Cholera Vaccine (Revised 1968) (Addendum 1973)
6. General Requirements for the Sterility of Biological Substances (Revised 1973)
17. Requirements for Inactivated Influenza Vaccine (Addendum 1973)
22. Requirements for Rabies Vaccine for Human Use

565 1975 WHO Expert Committee on Biological Standardization :

Recommendations for the Assessment of Binding-Assay Systems (including Immunoassay and Receptor Assay Systems) for Human Hormones and their Binding Proteins (A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems)

Development of national assay services for hormones and other substances in community health care

594 1976 WHO Expert Committee on Biological Standardization :

3. Requirements for Yellow Fever Vaccine (Revised 1975)
23. Requirements for Meningococcal Polysaccharide Vaccine
20. Specifications of tests used in the Requirements for *Brucella abortus* Strain 19 Vaccine (Live—for Veterinary Use) (Addendum 1975)

Annex 5

INTERNATIONAL STANDARDS AND INTERNATIONAL REFERENCE PREPARATIONS

NOTE: The lists of international biological standards and reference preparations previously included as annexes to the reports of the WHO Expert Committee on Biological Standardization are now issued as a separate publication.[1] Copies may be obtained direct, or through booksellers, from the agents listed on the back cover of this report, or they may be ordered from: World Health Organization, Distribution and Sales Service, 1211 Geneva 27, Switzerland.

The Expert Committee, at its twenty-seventh meeting, made the following changes to the lists already published.

Established:

Doxycycline	International Reference Preparation 1975
Neomycin	International Reference Preparation 1975
Minocycline	International Reference Preparation 1975
Human thrombin	International Standard 1975
Alphafetoprotein	International Standard 1975
Clostridium welchii (perfringens) beta toxoid	International Reference Preparation 1975
Clostridium welchii (perfringens) epsilon toxoid	International Reference Preparation 1975
Diphtheria toxoid, plain	Second International Standard 1974
Opacity	Fifth International Reference Preparation 1975
Anti-*Echinococcus* human serum	International Reference Preparation 1975
Anti-*Brucella abortus* serum	(The International Standard has now been given 1000 units of complement fixing activity)

Discontinued:

Fourth International Opacity Reference Preparation

[1] *Biological substances — international standards and reference preparations.* Geneva, World Health Organization, 1975. Revised editions incorporating the latest additions and amendments will be published every few years. The changes made between editions will be listed in the reports of the Expert Committee.

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Exhibit 9

Age-Related Disparity in Functional Activities of Human Group C Serum Anticapsular Antibodies Elicited by Meningococcal Polysaccharide Vaccine

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Serum bactericidal activity confers protection against meningococcal disease, but it is not known whether vaccine-induced anticapsular antibodies that lack bactericidal activity are protective. We developed an infant rat challenge model using a naturally occurring O-acetylated strain of *Neisseria meningitidis* group C and a strain that was negative for O acetylation (OAc). Rats 4 to 7 days of age inoculated intraperitoneally (i.p.) with $\sim 10^3$ CFU of either strain developed $>5 \times 10^5$ CFU/ml of blood obtained 18 h later. Dilutions of preimmunization sera given i.p. 2 h before the bacterial challenge had no effect on bacteremia, whereas group C anticapsular antibody in sera from adults immunized with meningococcal polysaccharide vaccine conferred complete or partial (>99% decrease in CFU per milliliter of blood) protection against the OAc-positive or OAc-negative strain, respectively, at antibody doses as low as 0.04 μg/rat. Anticapsular antibody at doses fivefold higher (0.18 to 0.2 μg/rat) in pooled sera from children immunized at a mean age of 2.6 years failed to protect rats, but antibody at the same or fivefold-lower dose in a serum pool from a group of children immunized at 4 years of age gave complete or partial protection. Protective activity was observed with some serum pools that lacked detectable complement-mediated bactericidal activity (titers < 1:4) and correlated with increasing antibody avidity. Thus, not only does the magnitude of the group C antibody response to meningococcal polysaccharide vaccine increase with increasing age but there are also age-related affects on antibody functional activity such that higher serum concentrations of vaccine-induced antibody are required for protection of immunized children than for immunized adults.

Neisseria meningitidis causes serious disease worldwide. In North America and Europe, the organism remains the most common cause of bacterial meningitis in children and young adults. Meningococci can be subdivided based on distinctive capsular polysaccharides. Isolates from five capsular groups—designated A, B, C, Y, and W135—are responsible for most cases of invasive disease. Thirty to forty percent of cases in North America and Europe are caused by capsular group C isolates.

The group C capsular polysaccharide consists of a homopolymer of α(2→9)-linked sialic acid (5, 31). In 90% of group C strains, the capsular polysaccharide is O acetylation (OAc) positive at positions 7 or 8, while in the remaining 10%, the capsular polysaccharide is OAc negative (3, 9). In OAc-positive strains, there is nonstoichiometric OAc of the capsular polysaccharide (thus, expression of both OAc-positive and OAc-negative epitopes) (31).

Meningococcal polysaccharide vaccines containing OAc-positive group C polysaccharide have been available for more than 30 years. These vaccines elicit serum antibodies largely in the absence of T-cell help (so-called thymic-independent antigens). In adults, these vaccines elicit high titers of group C complement-mediated serum bactericidal antibodies, and vaccination has been demonstrated to be highly effective in preventing disease (4, 11, 44). However, in infants and young children, the age groups at greatest risk of acquiring meningococcal disease, group C polysaccharide is poorly immunogenic (10, 34, 37) and is poorly efficacious (11, 48).

The presence of serum bactericidal activity remains the serologic hallmark of protective immunity against developing invasive meningococcal disease (6, 19). Although other immune mechanisms such as opsonic activity may also contribute to protection against meningococcal disease (12), it is not known whether sera that contain vaccine-induced anticapsular antibodies but lack complement-mediated bactericidal activity are protective against developing disease. To address this question, we developed an infant rat model of meningococcal bacteremia using either an OAc-positive or OAc-negative group C strain in order to correlate serum anticapsular and bactericidal antibody responses of children and adults vaccinated with meningococcal polysaccharide vaccine with the ability of the serum antibody to confer passive protection.

MATERIALS AND METHODS

Serum samples. We utilized serum samples that had been collected during two previous immunogenicity studies in children (see below), or from 17 laboratory workers with occupational exposure to meningococci and who had been immunized with Menomune (50 μg of A, C, Y and W135 polysaccharide per 0.5-ml dose; Aventis Pasteur, Swiftwater, Pa.) by Employee Health at Children's Hospital and Research Center at Oakland. The samples were stored frozen at −70°C. The adults provided written informed consent, and use of these sera for the present study was approved by the Institutional Review Board (IRB) of Children's Hospital Oakland and Research Center at Oakland.

One collection of stored sera from immunized children came from a study performed in 1982 in an Amish community of genetic factors affecting immune responses to polysaccharide antigens (24). Written informed consent was ob-

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tained from the parents of all children, and the protocol was approved by the IRB of Washington University School Medicine, St. Louis, Mo. Subjects in this study were given separate intramuscular injections of *Haemophilus influenzae* type b (Hib) polysaccharide vaccine (5 μg; vaccine prepared at the University of Rochester, Rochester, N.Y.) and meningococcal polysaccharide vaccine (10 μg of A and C polysaccharides in 0.1 ml; vaccine prepared by Connaught Laboratories, Swiftwater, Pa. [now Aventis Pasteur]). Although the doses used were one-fifth of the usual doses of these vaccines, the lower doses were in the immunogenic range (16, 22). Blood samples were obtained immediately before vaccination and 1 and 2 months later. The Hib anticapsular antibody responses measured in this study have been previously reported (24, 25). Since only small volumes of sera remained from this study, the sera used in the present study were selected from 17 children, ages 1.3 to 7.0 years, based on the largest quantities of sera available.

The second collection of stored sera were from subjects, ages 6 months to 19.9 years, immunized with meningococcal polysaccharide vaccine (Menomune; Connaught Laboratories) in eastern Ontario, Canada, as part of control measures instituted by the regional health departments during an outbreak of group C disease in Canada. The serum samples were obtained under a protocol approved by the IRB of the Children's Hospital Eastern Ontario, Ottawa, Ontario, Canada, and written informed consent was obtained from all participants or parents. The group C immunogenicity results in a random sample of 345 participants have been reported (29). For the present study, pre- and postimmunization sera from 31 children, ages 4.1 to 9.9 years, were selected randomly.

Serology. (i) RABA. The concentrations of group C anticapsular antibody were measured in the sera by a radioantigen binding assay (RABA) modified from that previously described (21). The group C meningococcal polysaccharide (provided by Aventis Pasteur) was derivatized with tyramine by a modification of the method of Lees et al. (30) and stored frozen in small portions at −70°C. On the week of the assay, an aliquot of antigen was thawed and the tyramine-polysaccharide was radiolabeled with ^{125}I by the chloramine T method (21). The average specific activity of the iodinated derivatives was approximately 2.5×10^7 cpm/μg of polysaccharide. The RABA was performed in 1.5-ml microcentrifuge tubes containing 50 μl of test sera which had been diluted with phosphate-buffered saline containing 5% (vol/vol) fetal calf serum (PBS-FCS) and an equal volume of radiolabeled polysaccharide that had been diluted in PBS-FCS to contain antigen at ~50 ng/ml (~80,000 cpm/50 μl). After incubation at 37°C for 2 h, 100 μl of 25% polyethylene glycol 8000 in PBS-FCS was added to the reaction mixture, mixed briefly with a vortex, and incubated overnight at 4°C. The following day, the precipitated immunoglobulin and immune complexes containing bound ^{125}I-labeled group C polysaccharide were harvested by centrifugation performed at 4°C for 10 min at 18,000 × *g*. The supernatant was removed, and the pellets were washed once in 0.5 ml of 12.5% polyethylene glycol 8000, and radioactivity in the precipitate were measured in a gamma counter. Antibody concentrations of the test sera were determined by comparison of the percentage of binding of the radiolabeled antigen by different dilutions of the test sample to that of a standard curve of binding at different dilutions of an anti-meningococcal serogroup A/C human reference serum (CDC1992, obtained from the National Institute for Biological Standards and Controls, Potters Bar, Hertfordshire, United Kingdom). This reference serum has been assigned a group C anticapsular antibody concentration of 32 μg/ml (27). As controls, each assay included a negative serum from an unimmunized adult with no detectable group C anticapsular antibody and three serum pools prepared from sera of immunized adults with low (0.4 μg/ml), medium (4.8 μg/ml), or high (44.8 μg/ml) anticapsular antibody concentrations.

All test serum samples were assayed in duplicate, and the respective pre- and postimmunization sera were measured in the same assay. For >95% of samples, bound polysaccharide in replicate dilutions of individual sera differed by less than 5%. To calculate interassay variation, the three serum pools described above were assayed on 12 occasions. The respective coefficients of variation for the results from these determinations ranged from 9.3 to 24%.

Anticapsular antibody avidity was measured in selected sera using a modification of the RABA originally described by Griswold et al. for measurement of Hib anticapsular antibody avidity (26). In brief, the total meningococcal group C antibody concentration was measured in each test serum by the RABA using radiolabeled group C polysaccharide at ~83 ng/ml of instead of the usual ~25 ng/ml in the final reaction vial. Also, incubation of serum and radiolabeled antigen was performed at 4°C for 18 h, instead of 37°C for 2 h as done in the usual RABA, described above. These changes in the protocol favored binding of both high- and low-avidity antibodies and provided a measurement of total antibody concentration, which in turn was used for calculation of the antibody avidity constant (Ka). The RABA was then repeated using a 25-fold-lower dose of radiolabeled antigen (3 ng/ml) to assure that the antibody concentration in the reaction vial was in excess, conditions that favor detection of primarily high-avidity antibodies. The fraction of bound antigen was determined at different antibody dilutions. The Ka was calculated by the following formula: Ka = fraction bound/{(1 − fraction bound) × [Ab]}, where fraction bound is the fraction of antigen bound at a particular antibody dilution, and [Ab] is the concentration of group C anticapsular antibody (molecular weight = 150,000) of the sample dilution tested, determined from the RABA using the high concentration of radiolabeled antigen (1). The avidity constant (nanomolar^{-1}) for each sample was calculated from the average Ka determined at antibody dilutions giving binding between 20 and 80%. For each test sample, the Ka was measured on at least two to four separate occasions. The assigned Ka represented the average Ka, omitting outlier results in the calculation.

(ii) ELISA. Immunoglobulin A (IgA), IgM, and IgG anticapsular antibody concentrations in serum were measured by an enzyme-linked immunosorbent assay (ELISA). The solid-phase antigen was adipic acid-derivatized polysaccharide, prepared from OAc-positive meningococcal C polysaccharide as previously described (23). The blocking buffer in the ELISA consisted of PBS-BSA (PBS with 1% [wt/vol] bovine serum albumin [BSA] [radioimmunoassay reagent grade; Sigma, St. Louis, Mo.]). Alkaline phosphatase-conjugated goat antibody specific for human IgA, IgM (Caltag Laboratories, Burlingame, Calif.), or IgG (Southern Biotechnology Associates, Inc., Birmingham, Ala.) antibodies were used as the secondary antibody detecting reagents. The ELISA employed replicate microtiter plates in which binding was measured using sera diluted with buffer alone or sera diluted with buffer containing soluble meningococcal group C polysaccharide (25 μg/ml) as an inhibitor. The antibody concentrations for the test sera were obtained by subtracting the respective absorbance values obtained with the sera diluted with the polysaccharide inhibitor from the corresponding values obtained with the samples diluted with buffer alone. The isotype-specific antibody concentrations in micrograms per milliliter were assigned to the test sera based on comparison to binding curves obtained with different dilutions of the CDC1992 reference serum, which has been assigned IgG, IgM, and IgA group C anticapsular antibody concentrations of 24.1, 2.0, and 5.9 μg/ml, respectively (27).

(iii) Inhibition of antibody binding by OAc-negative group C polysaccharide. For determination of the proportion of group C anticapsular antibody that was specific for epitopes expressed by OAc-negative polysaccharide, the ELISA described above was modified such that dilutions of sera were added to four replicate microtiter plates. The solid-phase antigen was an adipic dihydrazide derivative of the OAc-positive polysaccharide (23) that, because of nonstoichiometric OAc of the capsular polysaccharide, expressed both OAc-positive and OAc-negative epitopes. The presence of both structures was confirmed by nuclear magnetic resonance spectroscopy performed by Neil Ravenscroft, University of Cape Town, Rondebosch, South Africa. In one plate, the serum samples were diluted with buffer alone. In the remaining three plates, the sera were diluted with an excess of soluble OAc-positive meningococcal group C or OAc-negative meningococcal group C or, as a negative control, meningococcal group Y polysaccharides, respectively. The concentrations of inhibitor used in different experiments ranged from 10 to 25 μg/ml (10- to 25-fold higher than that needed for ≥90% inhibition of specific binding). The results of a typical inhibition experiment are shown in Fig. 1. Positive controls in the assay include two murine monoclonal antibodies (MAbs), MAbs 706 and 735, with known preferential binding to OAc-negative or OAc-positive polysaccharide, respectively (14). (Antibodies were kindly provided by K. Stein, U.S. Food and Drug Administration, Bethesda, Md. [now at MacroGenics, Inc., Rockville, Md.]). These MAbs were assayed in parallel with the human sera except that the secondary antibody used to detect binding of the MAbs was specific for mouse Ig (heavy and light chain; Southern Biotech) instead of human IgG. Binding of MAb 706 was typically inhibited by >90% in the presence of soluble OAc-negative or OAc-positive polysaccharide, with the inhibition by the OAc-positive polysaccharide reflecting nonstoichiometric OAc. Binding of MAb 735 showed significant inhibition only in the presence of OAc-positive polysaccharide. As a second positive control, we measured inhibition of binding of a postimmunization serum (serum 2C), which was obtained from an adult immunized with a group C polysaccharide-tetanus toxoid conjugate prepared from de-O-acylated group C polysaccharide by Baxter Health Care (Columbia, Md.) (38) (serum kindly provided by Ray Borrow, Meningococcal Reference Laboratory, Manchester, United Kingdom). With this serum, we typically observed >90% inhibition with OAc-negative or OAc-positive polysaccharide (Fig. 1), a result consistent with nearly all of the group C antibodies in this serum being specific for OAc-negative epitopes. For calculation of percent inhibition in test samples, absorbance values in the plate containing serum samples diluted with the OAc-positive meningococcal group C polysaccharide inhibitor were considered to represent nonspecific background binding and, therefore, were subtracted as background binding from the absorbance

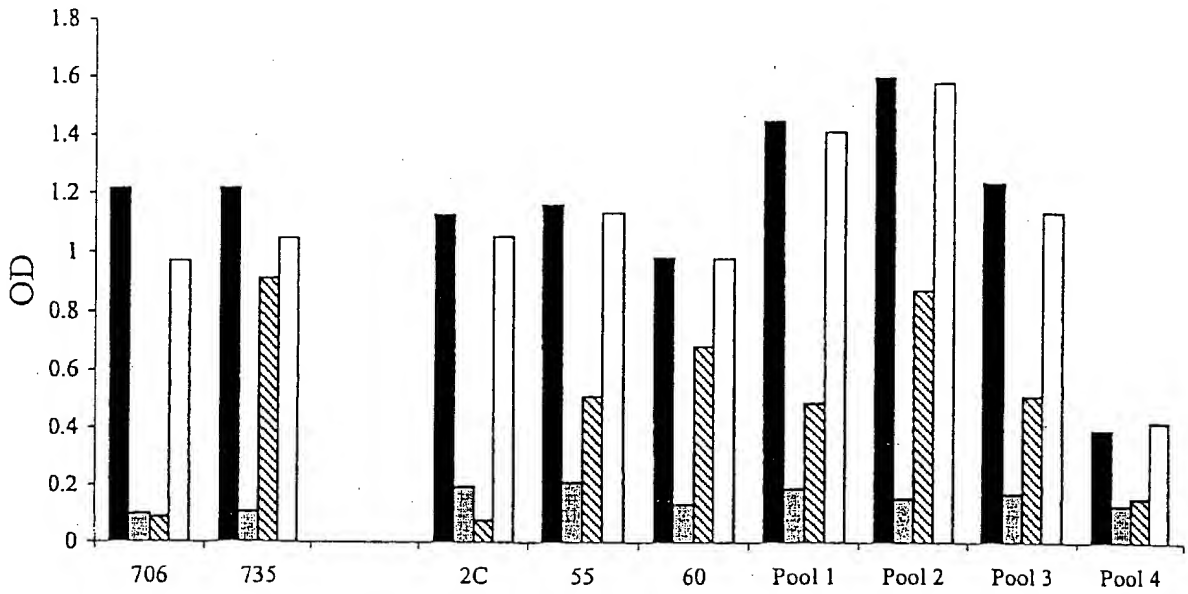

FIG. 1. Binding to solid-phase adipic dihydrazide derivative of OAc-positive polysaccharide, which is known to express both OAc-positive and OAc-negative epitopes. Sera were tested in the presence of a 10-μg/ml concentration of soluble OAc-positive (gray bars) or OAc-negative (striped bars) meningococcal group C polysaccharide or, as a negative control, group Y polysaccharide (open bars) (solid bars, no polysaccharide). The control MAb 706 is known to recognize an epitope on OAc-negative polysaccharide, but its binding is inhibited by both OAc-negative and -positive group C polysaccharides because of nonstoichiometric OAc. MAb 735 preferentially binds OAc-positive polysaccharide. The inhibition observed in the ELISA is consistent with this binding specificity. Human serum 2C from an adult immunized with the Baxter group C conjugate vaccine prepared from OAc-negative polysaccharide is completely inhibited by OAc-negative polysaccharide.

values of the respective serum dilutions incubated in the plate with no inhibitor. The resulting specific total absorbance reflected antibody populations specific for both OAc-positive and -negative group C polysaccharide. The percent inhibition by the OAc-negative meningococcal group C polysaccharide or the negative control group Y polysaccharide inhibitor was calculated from the equation {[(total specific absorbance) − (inhibited absorbance)]/(total specific absorbance)} × 100, using serum dilutions where the total absorbance (optical density) was approximately 0.9 to 1.4. The percent inhibition was calculated from the mean results from a minimum of two to three experiments performed on separate days. The negative control meningococcal group Y polysaccharide gave no significant inhibition of antibody binding to group C polysaccharide (Fig. 1).

(Iv) Complement-dependent bactericidal antibody activity. Bactericidal activity was measured as previously described (40) using log-phase test bacteria grown for approximately 2 to 2.5 h in Mueller-Hinton broth that had been supplemented with 0.25% glucose (wt/vol). Two group C meningococcal strains were tested—4243, which is naturally O-acetylated (OAc positive), and 4335, which is naturally not O-acetylated (OAc negative). These clinical isolates were provided by Trudy V. Murphy (now at Centers For Disease Control and Prevention [CDC], Atlanta, Ga.) and were collected during prospective surveillance of invasive meningococcal disease in Dallas County, Tex. (42). The two isolates were selected based on resistance to bactericidal activity of normal human serum and for their ability to cause bacteremia in infant rats (see below). The OAc status of these strains was determined by relative antibody binding as measured by a whole-bacterial-cell ELISA (40) using the OAc-positive specific murine MAb 1705.18, the OAc-negative specific MAb 181.1, and the backbone-specific MAb C2/1076.10 (14). The lack of OAc of the capsular polysaccharide purified from strain 4335 also was confirmed by nuclear magnetic resonance spectroscopy (kindly performed by Neil Ravenscroft). For measurement of bactericidal activity, all test sera were heated at 57°C for 30 min to inactivate intrinsic complement activity. The extrinsic complement source was serum from a healthy adult with no detectable anticapsular antibody to group C polysaccharide as tested by ELISA and no detectable intrinsic bactericidal activity against the target strains when tested at a final serum concentration of 20 or 40% (twofold higher than the serum concentration used for complement to test bactericidal activity in the test sera).

Passive protection against bacteremia in animal challenge model. The ability of group C anticapsular antibodies to confer passive protection against *N. meningitidis* group C bacteremia was tested in infant rats challenged intraperitoneally (i.p.). In development of the animal challenge model, we tested 12 group C clinical isolates for their ability to cause bacteremia in infant rats; these strains had been passaged in the laboratory fewer than three times. Of these, strains 4243 and 4335 gave the most consistent bacteremia with challenge doses as low as 500 CFU/rat. Subsequently, each of these strains was serially passaged three times in infant rats and stored frozen at −80°C in skim milk. Bacterial challenge of the rats was performed as previously described for group B strains (40). In brief, 4- to 7-day-old pups from litters of outbred Wistar rats (Charles River, Raleigh, N.C.) were randomly redistributed to the nursing mothers. On the day before challenge, freshly thawed bacteria were inoculated onto chocolate agar and grown overnight at 37°C in 5% CO_2. On the morning of the challenge, several colonies were inoculated into Mueller-Hinton broth supplemented with 0.25% glucose (wt/vol). After inoculation of the bacteria to a starting A_{620} of ~0.1, the test organism was grown for approximately 2 h with shaking at 37°C in 5% CO_2 to an A_{620} of ~0.6. After washing the bacteria twice in PBS-BSA, the bacterial suspension was diluted in PBS-BSA to contain approximately 10,000 CFU/ml.

At time zero, groups of animals were treated i.p. with 100 μl of different dilutions of test or control sera. Two hours later the animals were challenged i.p. with 100 μl of bacteria (500 to 1,000 CFU/rat). Heparinized blood samples were obtained by cardiac puncture 18 h later, and quantitation of bacteremia was performed by directly plating aliquots of 100, 10, and 10 μl of a 1:10 dilution (i.e., 1 μl) of blood onto chocolate agar. The number of CFU per milliliter of blood was determined after overnight incubation of the plates at 37°C in 5% CO_2. For calculation of geometric mean number of CFU per milliliter of blood, animals with sterile cultures were assigned a value of 1 CFU/ml, and animals whose samples produced a lawn of bacteria on culture plates streaked with 1 μl of blood were assumed to have more than 500,000 CFU/ml of blood and were assigned a value of 10^6 CFU/ml.

Statistical analysis. For calculation of geometric means, the bactericidal titers and anticapsular antibody concentrations of each subject were logarithmically transformed (base 10). Titers or concentrations below the lower limit of detection in the assay were assigned as half of the lower limit. For each age group, the mean of the log values of the antibody titers or concentrations, and the associated standard error (SE) of the means are reported along with the respective geometric means. In the passive-protection experiments, the proportion of animals with bacteremia and the respective geometric means of the numbers of CFU per milliliter of blood were calculated for each group of animals. The

TABLE 1. Group C serum antibody responses of adults and children immunized with meningococcal polysaccharide vaccine

Group[a]	No. of subjects tested	Mean age (yr) (range)	Mean log antibody concn (μg/ml) ± SE (geometric mean) as determined by:			
			RABA with:		ELISA with postimmunization sera and:	
			Preimmunization sera	Postimmunization sera	IgM	IgG
Adults	17	34 (18–45)	−0.43 ± 0.12 (0.4)	1.23 ± 0.11[b] (17.0)	0.23 ± 0.16 (1.7)	0.92 ± 0.20 (8.2)
Children						
Group 1	16	9.5 (8.2–9.9)	−0.63 ± 0.18 (0.2)	1.39 ± 0.14[b,c] (24.5)	−0.15 ± 0.25 (0.7)	1.18 ± 0.20 (15.1)
Group 2	15	4.5 (4.1–4.9)	−1.53 ± 0.10 (<0.1)	0.93 ± 0.07[c,d] (8.5)	−0.70 ± 0.32 (0.2)	0.86 ± 0.08 (7.2)
Group 3	5	4.6 (2.4–7.0)	−0.67 ± 0.39 (0.2)	1.03 ± 0.07[e] (10.8)	ND[f]	ND
Group 4	12	2.6 (1.3–4.8)	−1.39 ± 0.15 (<0.1)	0.37 ± 0.04[d,e] (2.4)	ND	ND

[a] Groups 1 and 2 were children immunized in a study in Canada (29). Groups 3 and 4 were Amish children immunized in a study in the United States (24). Preimmunization sera were obtained immediately before vaccination. Post-immunization sera were obtained 1 to 2 months later. IgM, IgG and bactericidal antibody responses were not performed on individual sera from Groups 3 and 4 because of insufficient quantities of sera (See methods).
[b] The geometric mean antibody concentrations of the adults and children in group 1 are not significantly different.
[c] The geometric mean antibody concentrations of groups 1 and 2 are significantly different ($P < 0.01$).
[d] The geometric mean antibody concentrations groups 2 and 4 are significantly different ($P < 10^{-6}$).
[e] The geometric mean antibody concentrations of groups 3 and 4 are significantly different ($P < 10^{-4}$).
[f] ND, not done because of insufficient quantities of sera.

significance of differences in the respective geometric means of the number of CFU per milliliter of blood from animals treated with different concentrations of group C anticapsular antibody were calculated using a two-tailed student t test (Excel software). For the purpose of determining the relationship between antibody avidity and passive protective activity, for each serum sample or serum pool, we determined the dose of antibody per infant rat required for a 2-$\log_{10}$ decrease in the number of CFU per milliliter of blood (i.e., 90% decrease), compared to that of control animals pretreated with preimmune serum or PBS. For the regression analyses, protective doses less than 0.008 μg/rat were assigned a value of 0.004 μg/rat and protective doses greater than 0.18 or 0.2 μg/rat (the highest doses tested for passive protection against the OAc-negative and -positive strains, respectively) were assigned a value of 0.4 μg/rat. The correlation coefficient, r, and the r^2 values between the Ka of each serum or serum pool and the respective $\log_{10}$ of the protective antibody dose were calculated by analysis of variance regression (polynomial degree = 2) (GB-STAT, version 6.5; PPC, Dynamic Microsystem, Silver Spring, Md.).

RESULTS

Age-related anticapsular antibody response. Table 1 summarizes the group C anticapsular antibody concentrations measured in serum samples obtained immediately before immunization and 1 to 2 months later. The results are stratified by the age of the subject at the time of immunization and the study in which the samples were obtained (groups 1 and 2 were from the Canadian study, and groups 3 and 4 were from the study done in an Amish community in Missouri). As expected (15, 37), the magnitude of the geometric mean serum anticapsular antibody concentrations increased with increasing age, being lowest in the 2-year-olds, intermediate in the 4-year-olds, and highest in 8- to 9-year-olds and adults. For all groups tested, IgG anticapsular antibody responses predominated with lesser concentrations of IgM anticapsular antibody.

Table 2 summarizes the serum bactericidal antibody responses measured against two group C test strains: 4243, which expresses O-acetylated capsular polysaccharide (OAc positive), and 4335, a strain that expresses capsular polysaccharide which is not O acetylated (OAc negative). The adults and the 8- to 9-year-old children showed significant increases in the geometric mean bactericidal titers after immunization. The respective postimmunization geometric mean titers of antibodies against the OAc-negative strain were approximately twofold higher than those measured with the OAc-positive strain. Three-quarters or more of the immunized adults or 8- to 9-year-old children had postimmunization bactericidal antibody titers of 1:4 or greater when tested against either group C strain. When measured with human complement, this threshold correlates with protection against developing meningococcal disease (6, 19, 45).

TABLE 2. Group C serum bactericidal titers of adults and children immunized with meningococcal polysaccharide vaccine

Group[a]	No. of subjects tested	Mean age (yr) (range)	Reciprocal titer of bactericidal antibody in serum					
			Strain 4243 (OAc positive)			Strain 4335 (OAc negative)		
			Mean log ± SE (geometric mean)		Post % ≥4[c]	Mean log ± SE (geometric mean)		Post % ≥4
			Preimmunization	Postimmunization		Preimmunization	Postimmunization	
Adults	17	34 (18–45)	0.30 ± 0.00 (2.0)	1.24 ± 0.17 (17.2)	76	0.38 ± 0.06 (2.4)	1.58 ± 0.22 (37.9)	76
Children								
Group 1	16	9.5 (8.2–9.9)	0.42 ± 0.07 (2.7)	1.14 ± 0.19 (13.7)	75	0.47 ± 0.10 (3.0)	1.48 ± 0.22 (30.1)	88
Group 2[b]	15	4.5 (4.1–4.9)	0.30 ± 0.00 (2.0)	0.36 ± 0.04 (2.3)	13	0.30 ± 0.00 (2.0)	0.67 ± 0.14 (4.7)	47

[a] Groups 1 and 2 were children immunized in a study in Canada (29). Bactericidal titers were not obtained for individual sera from children in groups 3 and 4 because of insufficient quantities.
[b] Children in group 2 had lower geometric mean bactericidal antibody responses than did the adults ($P < 0.002$) or children in group 1 ($P < 0.004$) for both the OAc-positive and OAc-negative strains.
[c] Percentage of individuals with postimmune bactericidal titers of ≥1:4.

TABLE 3. Group C anticapsular antibody concentrations of selected pre- and postimmunization sera or serum pools

Subject or pool no. (mean age [yr])	Total Anticapsular Antibody by RABA			Anticapsular Antibody by ELISA (postimmunization serum)			
	Concn (μg/ml) in serum		Mean Ka ± SE of postimmunization serum (nM^{-1})	Concn (μg/ml) of:			% IgG inhibited by OAc-negative polysaccharide[b]
	Preimmunization	Postimmunization		IgM	IgA	IgG	
Subjects							
54 (adult)	0.2	0.7	8.1 (6.8, 9.3)[c]	<0.5	0.2	<0.3	ND[d]
55 (adult)	0.1	48.8	26.4 ± 2.1	4.1	10.0	46.0	75
60 (adult)	0.8	55.8	25.0 ± 2.3	9.6	17.9	72.0	39
Pools[a]							
1 (9.5)	1.2	114	16.0 (14.0, 18.0)[c]	3.2	10.0	107	73
2 (4.5)	<0.1	13.9	20.3 ± 1.8[e]	0.2	2.4	16.6	54
3 (4.6)	ND	12.0	13.0 ± 0.8[e,f]	0.8	3.5	13.5	73
4 (2.6)	<0.2	2.8	7.0 ± 0.9[f]	<0.5	0.3	5.4	89

[a] Equal volumes of sera from each individual in groups 1 to 4 described in Table 1 were pooled to make pools 1 to 4, respectively.
[b] In an ELISA in the presence of soluble OAc-negative group C polysaccharide (10 to 25 μg/ml). The solid-phase antigen was adipic-dihydrazide-derivatized OAc-positive group C polysaccharide, which is known to express both OAc-positive and OAc-negative epitopes (see text).
[c] SE not calculated since only two independent measurements were performed. The values from each determination are given in parentheses.
[d] ND, not done because of insufficient concentrations of anticapsular antibody.
[e] $P < 0.02$ (for comparison of mean avidities of pools 2 and 3).
[f] $P < 0.01$ (for comparison of mean avidities of pools 3 and 4).

The bactericidal antibody responses of the children immunized at 4 years of age were much lower than those of the older children or adults. Only half of 4-year-olds developed titers of 1:4 or more when measured against the OAc-negative test strain, and only 13% developed titers of 1:4 or more against the OAc-positive strain. The low bactericidal antibody responses of the immunized 4-year-olds were unexpected since most of the children showed high anticapsular antibody responses to vaccination as measured by the RABA and/or IgG ELISA (Table 1), which used OAc-positive group C polysaccharide as the target antigen. The low or absent bactericidal responses despite the presence of relatively high serum anticapsular antibody concentrations imply that the anticapsular antibodies elicited by vaccination in these young children have poorer functional activity than those elicited by vaccination of older children or adults.

To investigate the protective activity of these antibodies in an animal challenge model, we pooled the respective pre- and postimmunization sera of children immunized at different ages (pre- and postimmunization pools 1, 2, 3, and 4 from groups 1, 2, 3, and 4, respectively, shown in Table 1). Table 3 summarizes the antibody concentrations measured in these serum pools and the results of characterization of the group C anticapsular antibody concentrations, isotype distributions, avidity, and the percentage of the anticapsular antibody response directed against the OAc-negative epitopes. For comparison, the corresponding results are shown for pre- and postimmunization serum samples from three representative control adults given meningococcal polysaccharide vaccine: subject 54, a low group C responder to vaccination, and subjects 55 and 60, who were chosen as representative of high responders to vaccination.

With one exception, the antibody concentrations measured in the serum pools were similar to the respective geometric mean antibody concentrations of the individual sera that made up the pools. The exception was the postvaccination serum pool from children immunized at 8 to 9 years of age (postimmunization pool 1), which had total and IgG anticapsular antibody concentrations of 114 and 107 μg/ml, respectively. The corresponding geometric means of the antibody concentrations of this group were lower, 24.5 and 16.9 μg/ml. These discrepant results were reproducible upon repeated assays. Each of the serum pools was prepared from equal volumes of sera from children in groups 1 to 4, respectively (Table 1). Sera from two individuals in group 1 had antibody concentrations more than 10-fold higher than the geometric mean antibody concentration of the group. Inclusion of these two outlier sera in the pool may have accounted for the discrepancy between the antibody concentration measured in the pool and the respective geometric mean antibody concentration of the group.

All of the children and adults in this study were immunized with vaccine prepared from OAc-positive group C polysaccharide. Nevertheless, with the exception of subject 60, 50% or more of the IgG group C anticapsular antibody binding in the ELISA was inhibited by soluble OAc-negative polysaccharide (Fig. 1 and Table 3). Previous work by Arakere and Frasch also demonstrated that a substantial amount of the serum anticapsular antibody in individuals immunized with a meningococcal polysaccharide vaccine was directed towards epitopes on the OAc-negative group C polysaccharide (3).

Table 4 summarizes the bactericidal antibody titers of the pre- and postimmunization serum pools and the corresponding titers of the three representative pre- and postimmunization sera from the adults. When tested against either group C strain 4243 (OAc-positive) or 4335 (OAc-negative), all preimmunization sera had bactericidal titers of <1:4 The postimmunization serum from subject 54, an adult known to be a low anticapsular antibody responder (Table 3), also lacked bactericidal activity. The postimmunization sera from the two high-responder adults (subjects 55 and 60), and the postimmunization serum pool from the children immunized at 8 to 9 years of age, had high titers of bactericidal activity against both group C test strains (Table 4). The postimmunization serum antibody concentrations required for 50% killing of the OAc-positive strain 4243 (BC_{50}) ranged from 1.1 to 2.3 μg/ml, which were approx-

TABLE 4. Serum bactericidal antibody responses of selected adults and children to meningococcal vaccination[a]

Subject or pool no. (mean age [yr])	Strain 4243 (OAc positive)			Strain 4335 (OAc negative)		
	Reciprocal titer in preimmunization sera	Postimmunization sera		Reciprocal titer of preimmunization sera[b]	Postimmunization sera	
		1/Titer[b]	BC_{50}[c]		1/Titer	BC_{50}[c]
Subjects						
54 (adult)	<4	<4	INDET[d]	<4	<4	INDET
55 (adult)	<4	21	2.3	<4	101	0.4
60 (adult)	<4	50	1.1	<4	170	0.1
Pools						
1 (9.5)	<4	100	1.1	<4	300	0.3
2 (4.5)	<4	<4	>3.5	<4	12	0.6
3 (4.6)	ND[e]	<4	>3.0	ND	<4	>2.2
4 (2.6)	<4	<4	INDET	<4	<4	INDET

[a] For description of pools, see footnote *a* of Table 3.
[b] Dilution of sera giving 50% killing in the presence of human complement. Note that all sera with titers of <1:4 were also negative (titer, <1:4) when retested with rat complement.
[c] BC_{50} for strain 4243 was calculated from the total antibody concentration measured by the RABA divided by the reciprocal bactericidal titer. A similar calculation was done to calculate the BC_{50} of strain 4335 except that the total anticapsular antibody concentration was adjusted to take into consideration the percentage inhibition by soluble OAc-negative polysaccharide (i.e., the portion specific for OAc-negative polysaccharide equals the total antibody concentration measured by RABA multiplied by the percent inhibited by OAc-negative polysaccharide in the ELISA (see Fig. 1).
[d] INDET, indeterminate because of low concentrations of anticapsular antibody.
[e] ND, not done.

imately 3- to 10-fold higher than the respective concentrations required for killing of the OAc-negative strain 4335 (Table 4). Note, for calculating the BC_{50} concentrations against the OAc-negative strains, we only considered the fraction of the serum anticapsular antibody concentrations that was specific for OAc-negative epitopes (i.e., inhibited in the ELISA by soluble OAc-negative polysaccharide).

The three postimmunization serum pools from children immunized before 5 years of age had no detectable bactericidal activity when tested against the OAc-positive strain 4243 (Table 4), and only pool 2, from the 4-year-olds immunized in Canada, was bactericidal for OAc-negative strain 4335 (titer = 1:12). The respective concentrations of anticapsular antibody required for 50% bacteriolysis in the postimmunization serum pools from children immunized before 5 years of age were two- to sevenfold higher than those for the serum pool from 8- to 9-year-olds, or for the individual sera from the two control adult responders.

Antibody avidity. One possible explanation for low bactericidal activity of the serum pools from the children is low antibody avidity. Figure 2 shows the ability of different concentrations of anticapsular antibodies in postimmunization serum pools 3 (mean age of children, 4.6 years) and 4 (mean age of children, 2.6 years) to bind to radiolabeled group C polysaccharide. For comparison is shown the corresponding binding of group C antibodies in the reference CDC1992 pool prepared

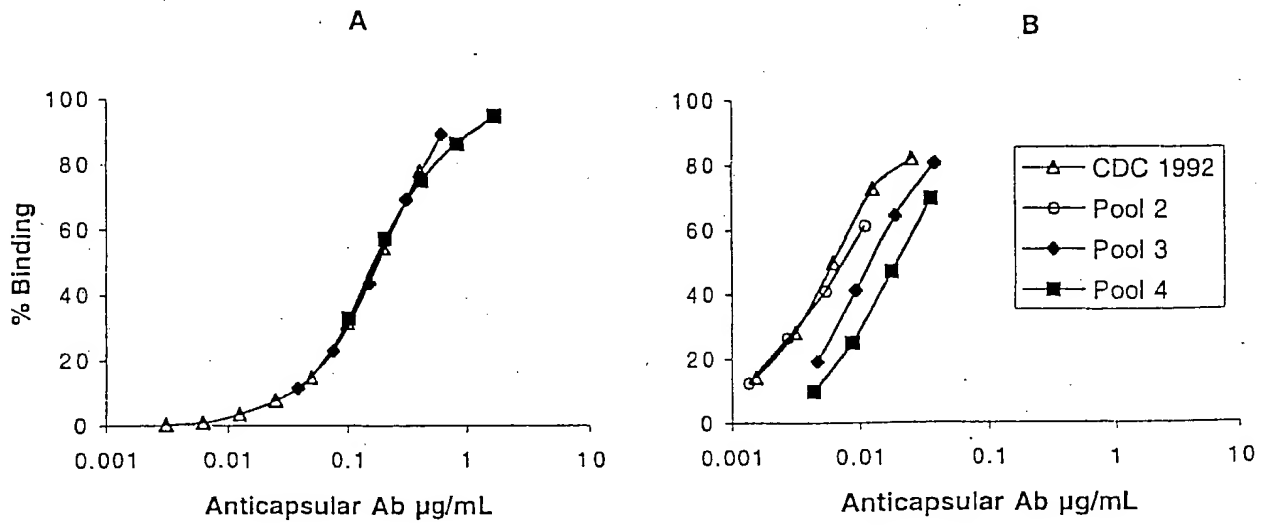

FIG. 2. Binding of anticapsular antibodies (Ab) to radiolabeled group C polysaccharide. (A) When a high antigen dose is used both high- and low-avidity antibodies are detected. (B) When a low antigen dose is used, detection of higher-avidity antibodies is favored. It takes four- to fivefold-higher concentrations of anticapsular antibody from pool 4, a low-avidity pool, to give equivalent binding to that of antibodies in the CDC1992 reference pool. The dose-response binding of pool 3 in the low-antigen assay is between that of pool 4 and that of the CDC1992 reference pool, and pool 3 is calculated to have an intermediate avidity Ka between that of pool 4 and that of the CDC pool (Table 3).

TABLE 5. Passive protection of infant rats challenged with group C *N. meningitidis* with adult sera

Subject no. and serum[b]	Dilution	Strain 4243 (OAc positive) (expt 1A)[a]			Strain 4335 (OAc negative) (expt 1B)[a]		
		Antibody dose (μg/rat)[c]	No. of animals positive for bacteremia/total no. tested	CFU/ml (geometric mean [10^3])	Antibody dose (μg/rat)	No. of animals positive for bacteremia/total no. tested	CFU/ml (geometric mean [10^3])
54							
Pre	1:2	0.01	5/5	>500	≤0.01	5/5	313
Post	1:2	0.04	5/5	>500	≤0.04	5/5	>500
55							
Pre	1:5	0.002	6/6	>500	≤0.002	3/3	>500
Post[d]	1:20	0.2	0/6	<0.001	0.14	3/5	0.897
Post	1:100	0.04	0/6	<0.001	0.03	5/6	21
Post	1:500	0.008	3/6	0.49	0.006	5/6	67
60							
Pre	1:5	0.02	6/6	>500	≤0.02	5/5	378
Post	1:28	0.2	0/7	<0.001	0.08	1/5	0.008
Post	1:140	0.04	1/7	0.004	0.02	5/6	3
Post	1:700	0.008	6/7	6.0	0.003	6/6	>500

[a] Experiments 1A and 1B were done on separate days. Results of experiments 2A, 2B, 3A, and 3B are shown in Table 6.

[b] Pre, preimmunization; post, postimmunization.

[c] The same respective dilutions of sera from each subject were tested against both strains. The antibody dose per rat for strain 4243 was calculated from the total antibody concentration measured by the RABA divided by the dilution. The antibody dose per rat for the OAc-negative strain 4335 was adjusted to take into consideration the percentage of antibody inhibited by soluble OAc-negative polysaccharide (see Fig. 1 and Table 4, footnote *c*).

[d] For subject 55, serum from 1 month postimmunization (39.5 μg/ml) was used for the passive-protection experiment with strain 4243. For the passive protection experiment with strain 4335, the serum from 2 months postimmunization was used because of insufficient quantity of the sample from 1 month postimmunization. Because the 2-month sample had a slightly higher antibody concentration than the 1-month sample (48.8 μg/ml), the 2-month serum was diluted 1:26, 1:130, and 1:650 to achieve doses of 0.2, 0.04, and 0.008 μg of total anticapsular antibody/rat, respectively.

from sera of adults given meningococcal polysaccharide vaccine (27). When a high radioantigen concentration is used in the assay (Fig. 2A), which detects both low- and high-avidity antibodies, the respective binding curves of the three pools overlap each other. In a separate experiment not shown, there was similar overlap of the respective binding curves of the CDC reference pool and pools 1 and 2 from the immunized children from Canada (mean ages of 9.5 and 4.5 years, respectively). In contrast, when a 25-fold-lower concentration of radioantigen was used in the RABA (Fig. 2B), conditions which favor detection of high-avidity antibodies (26), it took approximately four- to fivefold-higher concentrations of anticapsular antibody from pool 4 to give equivalent binding to that of antibodies in the reference CDC pool. With the low-antigen assay, binding of pool 3 was intermediate between that of pool 4 and that of the CDC1992 pool, and binding of pool 2 was indistinguishable from that of the CDC pool (Fig. 2B). The Ka ± SE calculated from these and other RABA data (not shown) were 7 ± 0.9 nM^{-1}, 13 ± 0.8 nM^{-1}, 20 ± 2 nM^{-1}, 16 nM^{-1} (SE not calculated because the sample was assayed only twice, with values of 14 and 18 nM^{-1}), and 17 ± 2 nM^{-1}, for pools 4, 3, 2, and 1 (Table 3) and the reference CDC1992 serum pool, respectively.

Passive protective activity in infant rats. Although the presence of serum bactericidal antibody correlates with protection against developing meningococcal disease, absence of bactericidal activity does not necessarily imply susceptibility to disease. To investigate this question in the pools of serum from the children immunized in this study, we adapted the infant-rat challenge model previously used for group B meningococci (40) to investigate passive protection against group C bacteremia. As negative controls in these experiments, a total of 41 infant rats were pretreated i.p. at time zero with 100 μl of 1:2 or 1:5 dilutions of sera obtained before immunization from four adults. Of the 41 animals, 23 were challenged i.p. with strain 4243 (OAc positive), and 18 were challenged i.p. with strain 4335 (OAc negative). For both strains the challenge bacterial dose used was between 500 and 1,000 CFU/rat. All 41 animals pretreated with preimmunization sera had bacteremia in blood obtained 18 h after the bacterial challenge (typically, 300,000 to >500,000 CFU per ml of blood).

Table 5 summarizes the results of pretreatment of rats with pre- or postimmunization sera from the three representative adults on the development of bacteremia 18 h after challenge with group C strain 4243 (experiment 1A) or 4335 (experiment 1B). None of the rats treated with 0.04 μg of antibody in postimmunization serum from subject 54, the control low responder adult with a bactericidal titer of <1:4, was protected against challenge by either strain (Table 5). However, the same dose of antibody in postimmunization sera from adult subjects 55 or 60 was highly protective against strain 4243, and a dose of 0.008 μg per rat gave partial protection against this strain. These data imply that there are important qualitative differences in anticapsular antibodies that may influence protective activity.

Table 6 shows the result of passive-protection experiments performed with serum pools from immunized children. Pool 1, which was prepared from serum samples of children immunized at a mean age of 9.5 years and which was highly bactericidal in vitro, was not tested for protection in the animal model since the purpose of these experiments was to determine whether sera from immunized younger children that lacked bactericidal activity could confer protection. Experiment 2A compared protection conferred by postimmunization

TABLE 6. Passive protection of infant rats with pediatric serum pools[a]

Expt no.	Subject or pool no. (age [yr]) and serum sample[b]	Dilution	Strain 4243 (OAc positive) (expt A)			Strain 4335 (OAc negative) (expt B)		
			Antibody dose (μg/rat)[c]	No. of animals positive for bacteremia/ total no. tested	CFU/ml (geometric mean [10^3])	Ab Antibody dose (μg/rat)	No. of animals positive for bacteremia/ total no. tested	CFU/ml (geometric mean [10^3])
2	Subject 55 (adult)							
	Pre	1:5	0.002	7/7	434	≤0.002	7/7[d]	>500
	Post	1:26	0.2	0/7	<0.001	0.14	0/7	<0.001
	Post	1:130	0.04	0/7	<0.001	0.03	3/7[d]	0.05
	Post	1:650	0.008	5/7	1.69	0.006	7/7	>500
	Pool 4 (2.6)							
	Pre	1:2	<0.005	7/7	>500	≤0.005	7/7	>500[f]
	Post	1:1.4	0.2	5/5	>500	0.18	3/3	49[f]
	Post	1:5	0.04	7/7[d]	>500	0.05	7/7	>500
	Pool 3 (4.6)							
	Post	1:2	0.6	0/5	<0.001	ND[e]	ND	ND
	Post	1:6	0.2	0/7	<0.001	0.15	3/7[e]	0.101
	Post	1:30	0.04	3/7	0.022	0.03	7/7	314
3	Negative control							
	PBS			5/5	>500		5/5	>500[g]
	Subject 55 (adult)							
	Post	1:26	0.2	0/5	<0.001	0.14	1/5	0.002
	Post	1:130	0.04	3/3	7.4	0.03	5/5	108
	Pool 3 (4.6)							
	Post	1:6	0.2	4/5	0.04[h]	0.15	5/5	23[g]
	Post	1:30	0.04	5/5	197	0.03	5/5	>500
	Pool 2 (4.5)							
	Pre	1:5	<0.02	5/5	>500	≤0.02	5/5	>500[g]
	Post	1:6	0.2	0/4	<0.001[h]	0.13	2/5	0.045
	Post	1:30	0.04	5/5	76	0.03	5/5	>500

[a] Experiments 2A and 2B and experiments 3A and 3B were done on separate days. For description of pools, see footnote *a* of Table 3.
[b] Pre, preimmunization; post, postimmunization.
[c] Antibody doses were calculated as described in footnotes *b* and *c* to Table 5.
[d] One rat in each of these groups was dead 18 h after bacterial challenge. These animals were presumed to be positive for bacteremia. For calculation of geometric mean CFU per milliliter, the dead animals were assigned values of 1,000,000 CFU/ml of blood.
[e] ND, not done.
[f] $P > 0.10$ (for comparison of geometric mean CFU per milliliter after treatment with postimmunization serum pool 4 to that of animals treated with preimmunization serum).
[g] $P = 0.05$ (for comparison of geometric mean CFU per milliliter after treatment with postimmunization serum pool 3 to that of the combined group of 10 animals treated with either preimmunization serum or PBS).
[h] $P < 0.03$ (for comparison of geometric mean CFU per milliliter after treatment with antibody (0.2 μg/rat) from postimmunization serum pool 3 to that of animals treated with antibody (0.2 μg/rat) from postimmunization serum pool 2).

serum pools obtained from Amish children immunized at mean ages of 2.6 (pool 4) and 4.6 (pool 3) years against challenge with strain 4243. Experiment 2B tested the same serum pools in rats challenged with strain 4335. Experiment 3A compared protection conferred by postimmunization pools obtained from two groups of children immunized at mean ages of 4.6 years (pool 3, U.S. Amish children) and 4.5 years (pool 2, Canadian children) against challenge with strain 4243. Experiment 3B tested the same serum pools in rats challenged with strain 4335. In experiments 2A and 3A, all 24 rats pretreated with preimmunization sera from the control adult 55 or pools made from preimmunization sera of children or with PBS had bacteremia following challenge with strain 4243 (>400,000 CFU/ml 18 h after challenge). Similarly, all 24 animals given these preimmunization sera and challenged with strain 4335 in experiments 2B and 3B developed bacteremia (geometric mean, >500,000 CFU/ml at 18 h after challenge). In both experiments 2A and 3A, a dose per rat of 0.2 μg of postimmunization serum from the adult positive control completely protected 12 of 12 rats challenged with strain 4243, and in experiments 2B and 3B, a dose per rat of 0.14 μg of the positive adult control serum completely protected 11 of 12 rats challenged with strain 4335. The respective results with the positive and negative control sera in these experiments were similar to those of experiment 1A and 1B (compare Table 6 and Table 5).

In experiment 2A, doses per rat of 0.2 and 0.04 μg of anticapsular antibody in postimmunization serum pool 3, obtained from Amish children immunized at an average age of 4.6 years, were completely or partially protective (>99% decrease in the number of CFU per milliliter of blood) against strain 4243. In contrast, comparable doses of antibody in pool 4 from Amish children immunized at a mean age of 2.6 years gave no pro-

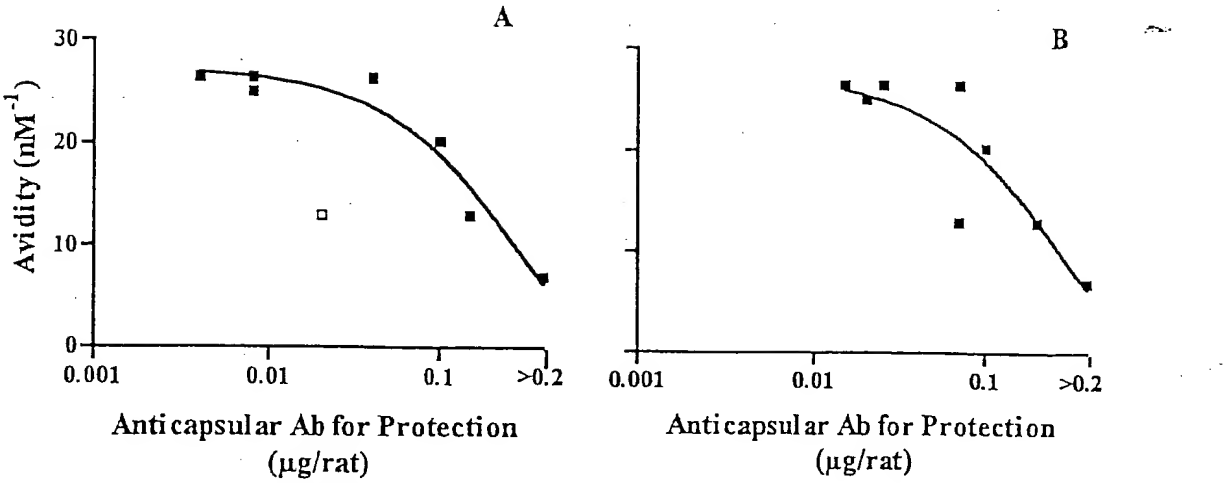

FIG. 3. Relationship between antibody (Ab) avidity and dose of antibody per rat required for passive protective activity (protective activity was defined as a 2-$\log_{10}$ decrease in the number of CFU per milliliter, compared to that of control animals pretreated with preimmunization serum or PBS). (A) Challenge by group C OAc-positive strain 4243. The point shown as an open square was considered an outlier and was excluded for purposes of the curve fit. (B) Challenge by group C OAc-negative strain 4335. The respective r^2 values calculated by analysis of variance nonlinear regression were 0.60 for strain 4342 ($P < 0.03$) and 0.69 for strain 4335 ($P < 0.01$). Excluding the outlier for strain 4243, the r^2 value was 0.92 ($P < 0.001$).

tection against this strain (Table 6). A similar trend was observed in animals pretreated with antibody in experiment 2B and challenged with strain 4335 (partial protection with a dose per rat of 0.15 μg of serum anticapsular antibody from the 4.6-year-olds but no significant protection at a dose per rat of 0.18 μg of serum antibody from the 2.7-year-olds. The postimmunization control serum antibody from adult 55 completely or partially protected against bacteremia caused by either strain at a dose of 0.03 to 0.04 μg per rat. Thus, there was an inverse correlation between age of immunization and dose of serum anticapsular antibody required to confer protection in this model. Also, some sera that lack bactericidal activity can be highly protective in vivo. For example, postimmunization serum pools 2 and 3 had no detectable bactericidal activity against strain 4243 (Table 4) but conferred protection against this strain in the animal model (Table 6). Postimmunization serum pools 2 and 3 also had no detectable bactericidal activity when tested with infant or adult rat serum as sources of complement; thus, passive protection by these serum pools may have resulted from a mechanism other than bactericidal activity such as opsonization.

In experiments 3A and 3B (Table 6), doses per rat of 0.13 to 0.2 μg of anticapsular antibody in postimmunization serum pool 2 from Canadian children immunized at an average age of 4.5 years were completely protective against strain 4243 and partially protective against strain 4335. This serum pool lacked bactericidal activity against strain 4243 and had a titer of antibody against strain 4335 of 1:12 (Table 3). Similar doses of antibody from pool 3, obtained from Amish children with a mean age of 4.6 years, were partially protective against strain 4243 but in this experiment gave minimal protection against strain 4335. Similar doses per rat of antibody from serum of the control immunized adult 55 completely protected against strain 4243 and partially protected against strain 4335.

Relationship between antibody avidity and passive protective activity in infant rats. Fig. 3 shows the relationship between the Ka calculated for each postimmunization control serum or serum pool tested for passive protection and the protective dose of anticapsular antibody determined for each of the passive-protection experiments. The protective antibody dose was defined as the dose per rat that gave a 2-$\log_{10}$ decrease in the number of CFU per milliliter, compared to that of control animals pretreated with preimmunization serum or PBS. Data from one sample (adult 54 with a Ka of 8.1 nM^{-1}) were excluded from the analyses since the highest antibody dose tested for protection of that serum was 0.04 μg/rat (Table 5) and, thus, the protective dose could not be defined. For both challenge strains, the higher the avidity of the serum anticapsular antibody, the lower the antibody dose required for conferring protective activity. The r^2 value calculated for the OAc-positive test strain 4243 (Fig. 3A) was 0.60 ($P < 0.03$), and that for the OAc-negative test strain (panel B) was 0.69 ($P < 0.01$). Excluding the one outlier (see legend to Fig. 3A), the r^2 value calculated for the OAc-positive test strain 4243 was 0.92 ($P < 0.001$).

DISCUSSION

The relationship between serum group C anticapsular antibody concentrations and protection from developing meningococcal disease is unknown. Young children immunized with meningococcal polysaccharide vaccine are reported to develop high serum group C anticapsular antibody responses as measured by ELISA (10, 37) or RABA (15, 16). Typically, however, there is little or no detectable bactericidal activity in this age group (10, 29, 37), particularly if measured with human complement (34). Our serologic results are consistent with these earlier observations. Children immunized at 2 to 4 years of age showed 20- to 80-fold increases in anticapsular antibody concentrations as measured by the RABA, comparing the respective geometric mean concentrations measured in sera obtained 1 to 2 months after vaccination to those in preimmuni-

zation sera (Table 1). However, the complement-mediated bactericidal antibody responses were poor (Tables 2 and 4), particularly when measured against the OAc-positive strain 4243; only 13% of the 4-year-olds developed titers of antibody to this strain of 1:4 or more after vaccination, a threshold predictive of protection against developing disease (6, 19). In experiments not shown, we also measured bactericidal antibody titers of sera from 22 immunized 4-year-old Canadians, including the 15 children in group 2, using two other OAc-positive strains, C11 (also called 60E) (19) and RM1090, and two other OAc-negative strains, 89I and 1088, as the target organisms. We obtained similar respective results as those shown for strains 4243 and 4335. Strain C11 is a group C strain used by many laboratories for measurement of group C serum bactericidal activity (19, 36, 45). Thus, the low bactericidal titers measured with the two group C test strains used in the present study are likely to be relevant to titers measured against other group C target strains expressing the OAc-positive or OAc-negative capsular polysaccharide.

Considerable data support a relationship between the presence of serum bactericidal antibody and protection from developing invasive meningococcal disease (reviewed by Frasch [13]). However, not all persons whose sera lack bactericidal activity are necessarily susceptible to developing disease (19). To date, this inference has not been tested experimentally because of lack of a suitable animal model of group C meningococcal disease.

The infant-rat challenge model described herein for group C bacteremia is similar to that used previously by us for investigation of the protective activity of antibodies to group B organisms (40). For the group C model, we use a relatively low challenge dose of 500 to 5,000 CFU of either an OAc-positive or OAc-negative strain. The challenge dose is prepared from organisms grown to mid-log phase in broth culture, and the bacteria are separated by centrifugation, washed, resuspended in buffer, and given i.p. In unprotected animals, the density of bacteria in the bloodstream increases within 18 h to >500,000 CFU/ml. Pretreatment of the animals with antibody can completely prevent bacteremia. Thus, the model permits investigation of the protective activity of group C anticapsular antibodies with different fine antigenic specificities related to OAc in an in vivo setting where, in the absence of protective antibodies, the organism is rapidly replicating.

Our most important findings from this model are as follows. (i) Serum anticapsular antibodies that are elicited by meningococcal polysaccharide vaccination of children, and which lack complement-mediated bactericidal activity, can confer protection. (ii) In the absence of bactericidal activity, a high antibody avidity correlates with in vivo protective activity. (iii) Age of immunization affects antibody functional activity. The serum samples used in our studies were obtained in previous studies of different populations (i.e., Canadian children and U.S. Amish children) using different vaccines (tetravalent or bivalent) and different vaccine doses. As described below, we do not believe these potential confounders affected the principal results or conclusions of this study.

In the Canadian study, all subjects received the same dose of tetravalent vaccine, and their sera were stored under identical conditions (−70°C). Within each age group, selection of the subset of sera used in our study was random since only a small subset of the samples had been assayed previously. Although immunization of the children was performed in response to an outbreak of group C disease, rates of disease in the population were low (~1/100,000 population [11, 29]). Also, colonization by group C strains is infrequent in the general population, even during outbreaks. Finally, the group C anticapsular antibody concentrations and bactericidal titers of the prevaccination sera of children in groups 1 and 2 were for the most part below detection (Tables 1 and 2), results consistent with lack of recent exposure to group C organisms. Thus, it is unlikely the antibody responses to vaccination of these subjects were affected by prior colonization by group C strains.

The Amish children in groups 3 and 4 received a bivalent meningococcal polysaccharide vaccine instead of the tetravalent vaccine, and one-fifth of the usual dose was given as part of a study of genetic factors affecting immune responses (24). However, both groups of Amish children were immunized in the same study, and the only difference between the two groups was the age of vaccination (mean age of 4.6 years for group 3 and mean age of 2.6 years for group 4). The serum pools from the two groups were compared directly in the same passive-protection experiments in infant rats (Table 6, experiment 2A, challenge by an OAc-positive strain, and experiment 2B, challenge by an OAc-negative strain). The results were unambiguous. Both serum pools lacked detectable complement-mediated bactericidal activity against both group C strains (Table 4). Nonetheless, the pool from the older children with higher antibody avidity (13 nM^{-1}) protected the rats from bacteremia at doses of 0.2 or 0.04 μg/rat, whereas the serum pool with lower avidity (7.0 nM^{-1}) did not confer protection at either dose. In the same experiment, the positive control serum from an immunized adult conferred protection at antibody doses of 0.2, 0.04, and 0.008 μg/rat. The superior protection of the adult serum correlated with the presence of complement-mediated bactericidal activity and also had higher-avidity antibody (26.4 nM^{-1}) than that of the serum pools from the two groups of children.

In experiments 3A and 3B (Table 6), we compared protective activity of serum pools prepared from two groups of children immunized at mean ages of 4.5 years (group 2, Canadian) and 4.6 years (group 3, Amish). The respective geometric mean pre- and postimmunization anticapsular antibody concentrations of the two groups were similar (Table 1), as were the respective total and IgG anticapsular concentrations of the pools prepared from the sera from the two groups. Although in this experiment there are many potential confounders (i.e., different vaccine, dose, and genetic backgrounds), the data confirm that serum pools without bactericidal activity against strain 4243 (pools 2 and 3) can be highly protective in the model. Also, the adult positive control serum with the highest antibody avidity (Ka = 26.4 nM^{-1}) gave the best protection, followed by the serum pool from group 2, which had a somewhat lower avidity (20.3 nM^{-1} [Canadian children]), with the lowest protection observed in animals pretreated with antibody from serum pool 3 from the Amish children, which had the lowest avidity (13 nM^{-1}). Thus, the results of the different passive-protection experiments with the different serum pools were consistent with respect to our findings that pools that lack bactericidal activity can confer protection, the finding of a correlation between increasing antibody avidity and increasing

protective activity, and the effect of older age of immunization and increased protective activity.

The RABA method used for measurement of antibody avidity in this study was adopted from the procedure originally described by Griswold et al. (26) in 1989 to measure the avidity of Hib anticapsular antibodies. In contrast to subsequently described ELISA methods that use chaotropic agents to dissociate antigen-antibody complexes (2, 17, 18) and give estimates of avidity (avidity index), the RABA method (Fig. 2) allows direct calculation of a Ka. Using this method, there was a good correlation between antibody avidity and the dose of serum anticapsular antibody required for protective activity (Fig. 3). Similar correlations between antibody avidity and anticapsular antibody functional activity have been observed for Hib (1, 33, 46) and *Streptococcus pneumoniae* (47, 49). Average affinity of the MAbs to phosphorylcholine also correlated with passive protective activity against experimental pneumococcal infection (41). However, in the absence of serum bactericidal activity against *N. meningitidis*, predicting protective antibody activity is still a complex undertaking. In addition to antibody concentration and avidity, the quality of the antibody can be affected by the isotype and specific epitopes recognized.

Our experimental data from the passive protection model are consistent with epidemiologic observations supporting protective efficacy of meningococcal vaccine when given to 4-year-old children (11, 44, 48), albeit less protective than in adults (11, 19, 20). The efficacy of group C polysaccharide vaccine in 2-year-old children is controversial, with some studies showing limited or no efficacy (11, 48). Although the experimental passive protection data reported herein for this age group are limited to sera obtained from only one group of Amish children immunized at 2 to 3 years of age, our results are consistent with the low vaccine efficacy in this age group (i.e., absence of both bactericidal and protective activity in pool 4).

Recently, meningococcal group C polysaccharide-protein conjugate vaccines were licensed in Europe and Canada (two prepared from OAc-positive polysaccharide and one prepared from OAc-negative polysaccharide). Following their introduction into routine and mass catch-up immunization in the United Kingdom, the number of cases of group C meningococcal disease has declined dramatically (28, 39, 43). The conjugated polysaccharide has T-cell-dependent antigenic properties. As such, these vaccines are more immunogenic in infants and young children than unconjugated group C polysaccharide, and the conjugate vaccines also elicit serum antibodies with greater avidity and bactericidal activity (6, 8, 10, 23, 32, 34, 35) and prime for memory antibody responses (7, 8, 35). In the future it will be of interest to investigate the protective activity of conjugate-vaccine-induced antibodies in the infant rat model and to dissect the relative protective role of antibodies elicited by OAc-positive or -negative polysaccharide.

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